Long-term change and genetic connectivity in the vegetation of mesotrophic semi-natural grasslands in the UK

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Contents

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Abstract

Species-rich grasslands have seen a drastic decline due to agricultural intensification and abandonment and this has resulted in a fragmented distribution of grassland sites. Such grasslands are highly diverse and are being managed for conservation. Long-term studies provide valuable evidence for the effective management of grasslands and this research investigated how the vegetation of species-rich grasslands in the Forest of Bowland, NW England has changed over 25 years, a period which has seen the introduction of statutory protection measures and agri-environment schemes. The study examined change in 35 grassland sites under contrasting management using data from two different survey methods. Results showed that overall community composition had remained stable but that change had occurred in the different management types. More detailed investigations were carried out on 14 sites which had been managed consistently as hay meadows. The meadow community had been maintained over 25 years but there were losses and gains of important species.

To investigate whether the changes in meadow plant populations were linked to the fragmented distribution of the sites an analysis of the genetic diversity within sites and gene flow between sites was carried out. Microsatellite markers were used to study the population genetics of *Rhinanthus minor* (Yellow Rattle), a key meadow species. It was found that there were moderate levels of genetic diversity and evidence of gene flow between the meadows, and between the meadows and intermediate grasslands including road verges. However, there were also local patterns of

differentiation. A comparison was made with samples collected from meadows in Worcestershire where there were similar levels of genetic diversity but less gene flow. These findings suggest that meadow management should be continued but should also include the wider landscape by creating or restoring other grasslands which can function as part of a network of sites.

Keywords: Grasslands; meadows; vegetation; connectivity; long-term change; conservation; population genetics; *Rhinanthus minor*; gene flow.

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Chapter 1. Introduction

Grassland habitats

Grassland habitats occupy around 40% of the earth's land surface (White *et al*., 2000) and exist within a broad range of environmental conditions. Grasslands are generally described as being dominated by grasses or grass-like plants (Dengler *et al*., 2013; Gibson, 2009) but some grasslands have a limited amount of tree cover or form part of a mosaic of other habitats. This means that they are less easily categorised than forest habitats which have well defined parameters such as the proportion of canopy cover or vegetation height (Dixon *et al*., 2014; FAO, 1998). Climatic influences are a key reason for the distribution of vegetation types (Box and Fujiwara, 2005), and dominance by grasslands is often correlated with drier conditions, particularly in the northern hemisphere where trees are less well adapted to drought (Bredenkamp *et al*., 2002). Examples of 'primary' or climax grasslands include the steppe vegetation of northern Europe and Asia whilst 'secondary' or plagioclimax grasslands, which often originated from cleared forest, are widespread in Europe (Kamp *et al.*, 2016; Hejcman *et al*., 2013).

In the temperate areas of Northern and Western Europe most grasslands can be referred to as semi-natural and are a result of human modifications which have halted succession to what would otherwise be forest or woodland habitats (Eriksson *et al.*, 2002; Tasser *et al*., 2007). Climax grasslands may exist in some areas such as in locations above the climatic treeline but even here they may be grazed by domesticated livestock or affected by other human influences. (Bredenkamp *et al*., 2002). Temperate European grasslands which have not been subject to intensive agricultural management are often described as 'semi-natural' in recognition of these human modifications but they support highly diverse vegetation (Habel *et al*., 2013; Poschlod and WallisDeVries, 2002).

Origins of semi-natural diverse grassland

Grasses and other plants that are part of present-day grassland communities existed in Europe long before the last period of glaciation (Kellog, 2001; Pärtel, 2005) but the extent of areas of open land within forested landscapes in North Western Europe in post glacial times has been debated for many years (Birks, 2005; Svenning, 2002; Vera, 2000). The role played by large herbivores in maintaining open areas within forests is included in this debate (Kuneš *et al*., 2015) and, in part, provides the context for current debates about the types and stocking densities of livestock in grasslands managed for conservation.

Humans began to make a significant impact on the landscape in the UK around 5,000 years ago, clearing forest for herding wild pigs and cattle but also for the cultivation of crops such as wheat and barley (Proctor, 2013). The first agricultural grasslands would have functioned as pastures and it is unlikely that hay making was carried out until metal implements were available during the Iron Age (Hejcman *et al*., 2013). In Europe the management of grasslands as meadows was thought to have been introduced by the Romans (Poschlod and WallisDeVries, 2002). Humans contributed to species diversity in grasslands through grazing their livestock, mowing, collecting firewood, the dispersal of seeds through local movements of people, livestock and hay; and grassland diversity was positively related to community size even 1000 years ago (Pärtel *et al*., 2007).

Change in grassland management and loss of semi-natural grasslands

The pattern of expansion and loss of extensively farmed grasslands varied in different parts of Europe (Hejcman *et al*., 2013; Poschlod and WallisDeVries, 2002) but in many countries it was in the 20th century that developments in agricultural management had a profound effect on the biodiversity of grasslands (Hodgson *et al*., 2005a; De Snoo *et al*., 2012). In the UK a shift in policy towards increased production, prompted initially by the goal of greater self-sufficiency during the Second

World War, led to the ploughing up of many meadows and intensification of other grasslands (Firbank *et al*., 2000; Hodgson *et al*., 2005a; Vickery *et al*., 2001).

A low-input, extensive management regime was replaced by a high input, more intensive approach to grassland management which could have a negative impact on the diversity of grassland vegetation for a number of reasons. Firstly, ploughing of grasslands would result in not only the loss of the existing vegetation but would limit any future regeneration because grassland species often have a transient seed bank (Pywell *et al*., 2002; Wellstein *et al*., 2007). Secondly, ploughing was usually followed by conversion to arable crops or re-seeding with a grass seed mix which was species poor and composed of competitive species with which the original plant community could not easily compete (Firbank *et al*., 2000). Thirdly, applications of artificial fertiliser would benefit the competitive species and lead to reduced species richness (Liira *et al*., 2012; Silvertown *et al*., 2006; Wesche *et al*., 2012). Fourthly, early and repeated mowing regimes, which were possible in fertilised grasslands, where silage production was the target crop, would prevent many species from setting seed. (Smith *et al*., 2000; Socher *et al*., 2012) Finally, increased livestock densities initially encouraged by the headage payments of the Common Agricultural Policy, would lead to decreased species richness because of impacts such as reduced ability for seed setting and raised fertility levels. (Fuller and Gough, 1999; Stewart and Pullin, 2008). An added problem in many European countries, and especially in mountainous regions, was that grasslands which were no longer economically viable were abandoned, and the lack of management resulted in scrub encroachment and eventually succession to a different habitat type (MacDonald *et* al, 2000: Poschlod *et al*., 2005; Tasser *et al*., 2007).

These changes to grassland management have been widely adopted in many European countries. In the UK as a whole it is estimated that there are approximately 193,500 hectares of lowland (i.e. enclosed grasslands below the moorland line) semi-natural grasslands (JNCC, 2017a). Blackstock *et*

al. (1999) reported an estimate for the extent of semi-natural lowland grassland in England and Wales as 50,000 to 100,000 hectares which equated to only 1–2% of the cover of permanent lowland grassland. Fuller (1987) calculated that approximately 97% of semi-natural grasslands (excluding rough grazing) had been lost in England and Wales between 1930 and 1984.

Grassland conservation

Legislation and policy measures to protect the remaining species-rich grasslands have been introduced. The EU Habitats Directive (92/43/EEC) at the European level and the Wildlife and Countryside Act (1981) at the national level are the key pieces of legislation which have enabled the statutory designation of grassland sites of conservation importance in the UK. Agri-environment schemes (AES) are implemented in many countries to establish management agreements with farmers and landowners who sign up to various conservation based options in return for payments (Batáry *et al*., 2015). In the UK the first AES were introduced in 1987 and there have been a number of revisions since then with the current Countryside Stewardship programme being launched in 2015 (Natural England, 2017a).

The efficacy of the measures which have been employed for the conservation of semi-natural grasslands and other habitats has been the subject of debate. In a review of protected areas in the UK, Gaston *et al*. (2006) found that information on their effectiveness was limited, and recommended that there should be: improved monitoring of protected sites with better use of baseline information; an evaluation of whether the current portfolio of sites was representative of key habitats and biodiversity features; greater understanding of the role of individual sites as part of functioning networks within the landscape matrix. The importance of a need for improved and better co-ordinated monitoring of protected sites in the EU Natura 2000 network has also been highlighted (Hochkirch, *et al*., 2013).

When protected semi-natural grasslands were compared with non-protected grasslands in the UK it was found that 91% of protected grasslands had retained their original grassland habitat compared with 27% of non-protected grasslands in the period from 1960-2013 (Ridding *et al.*, 2015). It seems that statutory site protection serves an important purpose in preventing the overall loss of conservation features but that protection could be more effective if detailed evidence was available about different habitat types, long-term and well-designed monitoring and consideration of the wider network of sites.

The concern about the lack of data on effectiveness was also raised with regard to AES by Kleijn and Sutherland (2003) who found that information on the evaluation of AES in several European countries was limited, but in schemes which aimed to enhance botanical diversity the outcomes were positive in less than half of the fourteen cases included in the study. Batáry *et al*. (2015) had access to data over a longer time period, and concluded that, in general, AES had been successful in their conservation objectives but that they could be better targeted, and that effectiveness had not improved over time despite the increased availability of scientific research and policy reviews aimed at better performance. Donald and Evans (2006) highlighted the fragmented distribution of many farmland habitats including semi-natural grasslands, and suggested that AES schemes should be designed to include the wider landscape to help counteract the impacts of fragmentation.

Key threats to semi-natural grasslands

There is no doubt that a low intensity management regime is essential for the protection and enhancement of vegetation diversity in semi-natural grasslands (Hansson and Fogelfors, 2000; Silvertown *et al*., 2006). Some of the wider environmental issues which will affect grasslands such as climate change and aerial nitrogen deposition have been addressed in studies considering a range of scales and approaches (Morecroft *et al*., 2009; Stevens *et* al, 2010; Van den Berg *et al*., 2011). It is clear that these anthropogenic impacts are on-going and the need for long-term studies to monitor

and analyse change in grassland vegetation, as well as the impacts on other taxonomic groups, is more pressing than ever.

The fragmentation of habitats is another of the key threats to biodiversity (Hanski 2005; Hanski, 2011), and lowland semi-natural grasslands in the UK are particularly vulnerable to habitat loss and isolation (Critchley *et* al., 2003; Hodgson *et al*, 2005a). The theory of island biogeography predicts that habit fragmentation would affect biodiversity because species richness is negatively related to habitat patch size and degree of isolation (Macarthur and Wilson, 1967). The effects of fragmentation on ecosystem functioning are complex (Fahrig, 2003) but may include changes in microclimate, alterations in edge effects, resource availability and such effects will vary according to the species as well as the size, shape and position in the landscape of the remnant habitat patches (Saunders *et al*., 1991). For plant populations fragmentation can inhibit pollen and seed dispersal leading to a decrease in gene flow which, in turn, may result in reduced genetic variation, increased inbreeding and increased genetic differentiation between populations (Leimu *et al.*, 2010; Young *et al*., 1996). The consequences of genetic isolation will vary from species to species (Debinski and Holt, 2000) but fragmented populations would be expected to be more susceptible to genetic drift and inbreeding depression, and ultimately to reduced fitness (Lienert, 2004).

Concerns about habitat fragmentation have led to the adoption of a landscape-scale approach to conservation (Donaldson *et al*., 2017) and in the UK the Lawton Report, *Making Space for Nature*, stressed the importance of connectivity between habitats and the need to improve the wider environment around protected sites as well as managing for the quality and size of habitats (Lawton *et al*., 2010). If enhancing connectivity between habitats is to be effective then research which provides empirical evidence of the extent of genetic variation within, and gene flow between, semi-natural grasslands will be of value for the design of conservation strategies.

Mesotrophic semi-natural grasslands and hay meadows

Semi-natural grasslands support many different vegetation communities and their composition can be influenced by numerous factors including soil type and underlying geology, climate, hydrology, altitude, proximity to the coast and the management regime (JNCC, 2017b). Calcareous grasslands support some of the most diverse vegetation in the world (Poschlod and WallisDeVries, 2002) but grasslands on circum-neutral soils, also known as mesotrophic grasslands, are often the most vulnerable to agricultural intensification and yet are less well-studied than other grassland types (Vickery *et al*., 2001). Mesotrophic grasslands may be managed by grazing or mowing and, in common with other semi-natural grasslands, species diversity is maintained by low intensity management (Critchley *et al*., 2007; Stewart and Pullin, 2008). In terms of area hay meadows are the most poorly represented enclosed semi-natural grassland type in the UK and upland hay meadows are particularly scarce with only 900 hectares remaining (JNCC, 2017a). In the EU as a whole there are only around 2000 km^2 of mountain hay meadow habitat, a category which includes several of the study sites included in this thesis (Rodwell *et al*., 2013). This means that studies which investigate long term change and fragmentation in these habitats will be adding to the evidence base for the conservation of a rare and vulnerable habitat.

Data from long-term grassland experiments such as the Park Grass Experiment and the Steinach Grassland Experiment have provided important temporal information about the different effects of management on small scale grassland plots (Hejcman *et al*., 2014; Silvertown *et al*., 2006). Re-visitation studies, in which surveys are repeated after a period of several years, or even decades, add to this information by examining long-term change in grasslands in the wider landscape (Diekmann *et al*., 2014; McGovern *et al*., 2011). Long-term change in mesotrophic grasslands is less well studied and there appear to be no studies of long-term change in hay meadows managed for conservation in the 25 years since AES were introduced in the UK. This study will address that gap in the literature by investigating long-term change in mesotrophic grasslands and using the genetic analysis of a key meadow species to explore the impact fragmentation might have on changes in grassland

populations. The findings from the study will be of value beyond the UK because the effectiveness of the conservation management of meadows, and the impact of fragmentation are of concern to ecologists and conservation managers of mesotrophic grasslands wherever they are located.

Research aims

This thesis will take a novel approach to the analysis of change in the community composition of semi-natural grasslands by combing a long-term study with an analysis of genetic variation and gene flow. The aims of the study are: to investigate how vegetation has changed in mesotrophic grasslands with a particular focus on hay meadows, and to discover what might be the drivers for this change; to analyse whether the fragmented distribution of meadows has affected the genetic diversity within, and gene flow between, meadow populations; to establish how the patterns of genetic variation in the study area compare with a contrasting study region; to propose how the findings of the study could be applied to the conservation of hay meadows.

Thesis outline

Chapter 2 will investigate change over 25 years in the community composition of the vegetation of 35 mesotrophic grasslands in the Forest of Bowland, North West England, with contrasting management regimes by comparing survey data from the 1980s with repeat surveys from 2012 and 2013. These data are from quadrat surveys of the principal vegetation communities and species list surveys of the whole grassland site.

Chapter 3 will look in more detail at long-term change in 14 sites which were consistently managed as hay meadows. This chapter will investigate the differences in cover of key meadow indicator species and the possible drivers for change in community composition.

Chapter 4 will assess the impact of the fragmented distribution of protected meadows on genetic diversity within, and gene flow between, meadow sites in the Forest of Bowland using the results of microsatellite marker analyses of the key meadow species, *Rhinanthus minor*.

Chapter 5 will draw a comparison of genetic variability and gene flow patterns in meadow populations of *R. minor* sampled in Worcestershire, a lowland area of the UK, with the results of the Bowland study.

Chapter 6 will set out the overall conclusions of the thesis and will describe their implications for the conservation of semi-natural grasslands.

Nomenclature for vascular plants follows Stace (2010). Plant name authorities are cited in Appendix 2.

Chapter 2. Regional stability versus fine scale changes in community composition of mesotrophic grasslands over 25 years.

This chapter has been published in the *New Journal of Botany*, Volume 7 (2017). A copy of the published version can be found at Appendix 5.

Introduction

Species-rich grasslands support a rich diversity of vegetation but they are particularly vulnerable to change (Habel *et al*., 2013). In Europe the maintenance of diversity in such grasslands usually requires an extensive agricultural management regime (Wesche *et al.,* 2012), so the widespread adoption of intensive agricultural practices and the abandonment of more inaccessible or unproductive grasslands has resulted in the loss of the majority of species rich grasslands in most European countries (Stoate *et al*., 2009). Studies which record long-term change in the remaining species rich grasslands are rare but can make a valuable contribution to our understanding of ecological processes as well as helping to inform management approaches (Magurran, *et al.*, 2010; Silvertown *et al.*, 2010).

Some of the most diverse vegetation communities are found in calcareous grasslands and these habitats have been the subject of studies concerning change resulting from various influences and at different scales (Bennie *et al.*, 2006; Diekmann *et al.*, 2014; Van den Berg *et al.*, 2011). Although some attention has focused on change in hay meadows (Critchley *et al.*, 2007; Homburger and Hofer, 2012), mesotrophic grasslands are less well studied, particularly those managed as pasture (Stewart and Pullin, 2008). However, mesotrophic sites can be botanically rich and may be more vulnerable to agricultural intensification than calcareous grasslands because of their higher levels of soil fertility (Hodgson *et al.*, 2005). The management of semi-natural mesotrophic grasslands is often dependent on topography with grazing dominating sites with steeper slopes whilst mowing for field-dried hay

will be carried out on flatter ground (Andrieu *et al.*, 2007). Community composition of grassland vegetation varies according to management type (Klimek *et al.*, 2007) so studies which consider sites under different management regimes provide additional important information about long-term change.

Long-term, experimental studies such as the Park Grass experiment and the Steinach Grassland experiment provide detailed, temporal data about different management treatments in grassland systems (Hejcman *et al*., 2014; Silvertown *et al*., 2010). Other approaches to investigating long-term change include re-visitation studies which consider various types of sites sometimes located over a large geographical area. Such studies deliver valuable complementary information to that generated by the monitoring of experimental plots and provide an indication of change over a wider spatial scale. Re-visitation studies have revealed widespread change such as the effects on species richness in coastal vegetation communities around Scotland (Pakeman *et al*., 2016) and a loss of distinctive species in calcareous grasslands in sites across the UK (Bennie *et al*., 2006; Van den Berg *et al.*, 2011). There are fewer studies of mesotrophic grasslands but Critchley *et al* (2007) found a reduction in herb cover in species rich hay meadows at a regional scale.

The present study investigated change in 35 mesotrophic grassland sites first surveyed in the 1980s and 1990s by the UK Nature Conservancy Council. The grasslands included sites which had been consistently managed as either hay meadows or as pastures. It also included sites originally managed as meadows but which had seen a change to more intensive management, and sites where there was no management or only occasional management. It would be expected that a change to more intensive management or to a lack of regular management would be more likely to result in corresponding changes in community composition, a relationship which has been widely discussed (Hodgson *et al.*, 2005; Krause and Culmsee, 2013; Peco *et al.*, 2005; Poschlod *et al.*, 2005).

Re-visitation studies often use quadrat surveys to repeat previous vegetation surveys (Critchley *et al.* 2007; Meyer *et al.*, 2015; Ross *et al.*, 2012). Quadrats enable a standard, repeatable survey method although there is debate about optimum quadrat size and about inconsistencies in the estimation of percent cover (Archaux *et al.*, 2007; Kent, 2012). In the present study repeat quadrat surveys were carried out but baseline data was also available for site species lists for all of the sites included in the study. Whilst the quadrat data account for the principal vegetation communities, whole site species lists can reveal information about the vegetation in atypical parts of a grassland site such as ditches, wetter areas and sloping banks which were often less accessible to livestock or machinery and which can enhance the diversity of the vegetation across the site.

The statistical analysis of data obtained from studies which use stratified random sampling or quadrats placed subjectively in representative stands of vegetation will be more limited than that of data obtained from using an entirely random sampling design (Lajer, 2007). However, it is recognised that there is considerable value in the data from the numerous relevés which have been recorded over many years as part of phytosociological and other vegetation studies, provided that it is analysed and interpreted appropriately (Diekmann, *et al.,* 2007; Hédl, 2007; Lepš and Šmilauer, 2007). A similar approach should be taken with data collected from site species lists which can also be affected by surveyor bias but which can provide important information particularly where resources for surveys are limited (Gordon and Newton, 2006).

This study explored long-term change in the vegetation of 35 mesotrophic grasslands located across an upland region of north-west England. Unlike other revisitation studies it combined a comparison of long-term change in grasslands with different management regimes and used data from two different survey methods.

The study addressed the following questions:

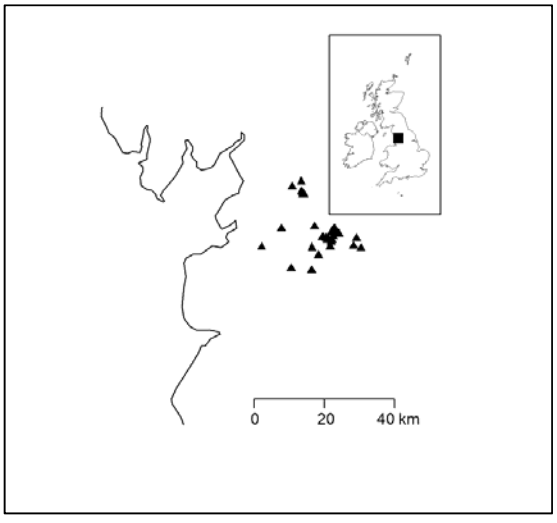
1. Has the overall community composition of grassland vegetation changed?
2. Are there differences in the extent of change between grasslands with different management types?
3. Do the two survey methods provide contrasting information about vegetation change?
4. Which species are the main 'winners' and 'losers'?

Methods

Study area

The study was carried out in the Pennine region of North West England. The study sites were located within an area of approximately 450 km^2 in the valleys of the Forest of Bowland which is an upland area situated at 53°58′N, 2°26′W (Fig. 2.1). The mean annual precipitation for the region is 1294mm, mean January temperature is 4.0°C and mean July temperature is 15.8°C (Met Office, 2016).

Fig. 2.1 Location map



Site selection

35 grassland sites were identified where baseline survey data for both quadrat and species list surveys were available. The data had been collected in the 1980s and 1990s as part of a UK wide grassland survey (Blackstock *et al*., 1999). Part of this nationwide survey focused on mesotrophic lowland (i.e. below the moorland line or lower than approximately 300m above sea level) grasslands in Lancashire and it is this dataset that forms the baseline for the present study (Taylor, 1986.). Grasslands in the original surveys were selected using existing Phase 1 habitat survey records and other local information and were chosen because they were species rich or moderately species rich. The surveys aimed to record sites which were important for conservation and to compare the botanical detail of sites with similar vegetation classifications.

The study incorporated sites from the Forest of Bowland region with contrasting management regimes including fourteen sites which had been managed continuously as hay meadows since the original surveys were undertaken. Management details for these sites such as earliest cutting dates, amounts of farmyard manure and dates of removal of grazing stock in the spring can be linked to their inclusion in agri-environment schemes or designation as protected sites. Ten sites had been managed by grazing (cattle, sheep or a mixture of both). There were also six sites which were hay meadows at the time of the first survey but which had seen a change in management since the first survey was undertaken. The timing of the change is not known for all of the sites but the current management is more intensive and involves either permanent grazing or cutting for silage rather than hay. The remaining five sites are no longer regularly cut or grazed but, again details of the timings of the change are not known for all of the grasslands. The sites were located at altitudes varying from 60m to 280m above sea level. Sites varied in size from 0.2ha to 11.59 ha (Table 2.1).

In the original surveys the grasslands the grasslands were classified under the UK National Vegetation Classification (NVC) as upland hay meadows MG3 *Anthoxanthum odoratum-Geranium*

sylvaticum, floodplain meadows MG4 *Alopecurus pratensis-Sanguisorba officinalis* and lowland hay meadows or pastures MG5 *Cynosurus cristatus-Centaurea nigra* communities (Rodwell, 1992) although the majority of the surveys took place before the NVC was published and none of them were part of the NVC survey itself. These are the main communities but some grasslands would also have supported or still support small areas of other mesotrophic examples. Most of the grasslands belong to the Triseto-Polygonion alliance or are associated with alliances within the Molinio-Arrhenatheretea order (Rodwell *et al*., 2007).

Table 2.1. Site details

Site ID	Management type	Size (ha)	Altitude	No. of quadrats
AM	former meadow	1.8	185	3
BG	meadow	5.47	180	1
BG2	meadow	2.2	180	1
BG3	meadow	3.1	180	2
BS (3 fields)	meadow	7.65	150	12
BS1	meadow	2.3	180	2
BS3	grazed	1.2	170	3
CB	meadow	0.54	60	4
DH	meadow	0.4	190	2
FH	meadow	1.63	105	2
FHM	meadow	3.33	210	4
HHL	unmanaged	10.3	195	6
HHM	unmanaged	0.3	105	2
LBL	former meadow	1.7	140	2
LCB	grazed	6.0	180	3
LCM	meadow	5.26	190	2
LHBS	grazed	0.76	130	2
LHG	grazed	2.2	100	3
LRS	unmanaged	0.2	120	2
LSM	former meadow	1.1	230	1
LWM	unmanaged	3.6	105	2
MM (2 fields)	meadow	9.09	155	9
MM2	grazed	0.7	160	3
NI	meadow	2.09	125	6
NKM	grazed	3.9	180	6
OWP	grazed	0.3	160	6
PHB	unmanaged	0.5	135	2
PP	grazed	1.8	150	10
RH	former meadow	1.8	80	2
SFP	grazed	4.5	230	11
SM	meadow	3.63	200	2
SPM	grazed	1.4	280	6
TB (5 fields)	meadow	11.87	155-180	7
TL	former meadow	0.4	220	2
TSM	former meadow	6.4	185	3

Fig. 2.2 Example of grazed site

Fig. 2.3 Example of meadow site

Data collection

Repeat surveys (hereafter the second survey) were carried out using the original methods in the summers of 2012 - 2014. The original surveys (hereafter the first survey) followed Nature Conservancy Council guidance and involved the placing of 1x1m quadrats in areas deemed to be representative of the main vegetation communities (Smith *et al.*, 1985). The guidance stated that the quadrats should be placed randomly within each vegetation community although it was acknowledged that this would not always be possible, particularly in smaller stands of vegetation. In the meadow communities a random sampling approach would be straightforward but this might not have been achievable in some of the grazed sites where species rich flushes and other smaller vegetation stands where surveyed.

Sketch maps of the locations of the first survey quadrats (see Smith *et al.,* 1985 for an example map) were used to locate the quadrats in the second survey. The placing of the second survey quadrats followed the original approach by selecting areas representative of the main communities using the sketch maps and detailed descriptions of the vegetation to ensure they were in the correct area of the site. The number of quadrats varied depending on the size and complexity of the sites (Table 2.1). In the meadows quadrats were estimated to be within approximately 25m of the original location although this would vary according to the size of site and number of quadrats. The grazed sites were often more variable with a mosaic of various vegetation communities which accounted for the higher number of quadrats in the original surveys, but in these sites the re-location of the quadrat was aided by descriptions of particular vegetation stands such as a species rich flush or by proximity to a feature such as a stream. Presence and abundance, using the Domin scale, of all vascular plants were recorded.

In addition to the quadrat surveys the first survey involved the compilation of a species list covering all areas of the site on and within the site boundary (so vegetation in boundary hedges was included). Site boundaries included hedges, dry stone walls, post and wire fences and watercourses or ditches. None of these boundaries had been removed or re-positioned since the first surveys were undertaken. The NCC guidelines did not require surveyors to time the species list survey but there was a requirement to include all of the vegetation communities on the site.

Data analysis

An exploratory approach to data analysis was taken because it could not be assumed that random sampling methods had been used for all of the quadrats or for the collection of species list data. To analyse differences in community composition Non Metric Multi-dimensional scaling (NMDS) ordinations were undertaken on the first and second surveys quadrat and species list data. The Domin scores recorded in the quadrat surveys were converted to percentage values by using midpoint of

each Domin category. The Bray Curtis dissimilarity matrix was used and the NMDS ordinations were carried out using the metaMDS function in the vegan package in R (Oksanen, *et al.,* 2013). The NMDS ordinations examined community composition by year and then separate ordinations were carried out on the quadrat data to investigate the four management types, i.e: meadows, grazed sites, former meadows with more intensive current management, and sites which had little or no management.

To investigate patterns in community composition revealed by the two survey methods (quadrat surveys and species list surveys) the quadrat data were first converted from abundance data to presence/absence data so that they were analysed in the same format as the species list data. NMDS ordinations for the two survey types were then compared using Procrustes analysis in the vegan package (Oksanen *et al*., 2013). Procrustes analysis is used to investigate the extent to which there is a fit between one ordination or dataset and another and produces a correlation score indicating the extent of the fit based on the distances between the sampling points or sites. A low score would indicate that there was little similarity between the two ordinations and *vice versa*. Protest does return *P* values but large datasets can affect the validity of *P* values and it is recommended that the *r* value is more useful in interpreting the outcome of the test (Oksanen, *et al.,* 2013).

To analyse species losses and gains, species were ranked according to the frequency at which they had been recorded by site in the first and second surveys in both quadrats and species lists.

In the UK guidance is issued for the monitoring of protected mesotrophic grassland sites (JNCC, 2004). The guidance lists species for each grassland community which are considered as positive indicators whose presence is indicative of favourable conservation status. These indicator species are used to evaluate the conservation value of particular grassland communities and to address whether the target vegetation community is being maintained or not. The frequency of positive and negative

indicator species by site were compared for the first and second surveys. Indicator species are listed in Appendix 1.

To assess whether there was any indication of change in functional type in the increased and decreased species mean values for Ellenberg Indicator Values (EIVs) for the British plants for light (L), moisture (F), reaction (R) and fertility (N) were calculated for the most increased and decreased species (Hill *et al*., 1999). Weightings were not used for the EIVs because there were no abundance data for the species lists. Ellenberg values can give an indication of changes in environmental conditions and are useful as a proxy measure when no soil data is available as was the case for these surveys. Calculations of Grime's C-S-R plant strategy scores using the tool developed by Hunt *et al.* (2004) were also undertaken and assigned to the most increased and decreased species. The modal C-S-R type was calculated.

All analysis was carried out in R version 3.1.2 (R Development Core Team, 2014).

Results

In the quadrat survey the total number of species recorded across all 35 sites was 152 from the first surveys and 144 from the second survey (a decrease of 5.26%). In the species list survey the totals were 268 from the first survey and 229 from the second survey (a decrease of 14.55%).

The NMDS ordination plots do not show a distinct separation of survey sites by year for either the quadrat data or the species list data (Figs. 2.4a and 2.4b) indicating that there is little difference in overall community composition between the two survey years.

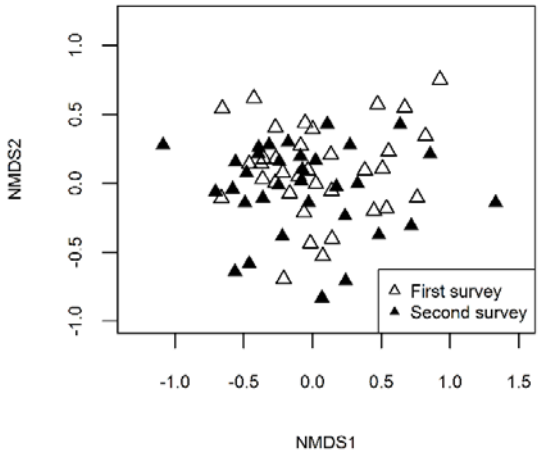


Fig. 2.4a NMDS ordination of quadrat data for the first and second surveys. Points represent grassland sites. Stress = 0.22

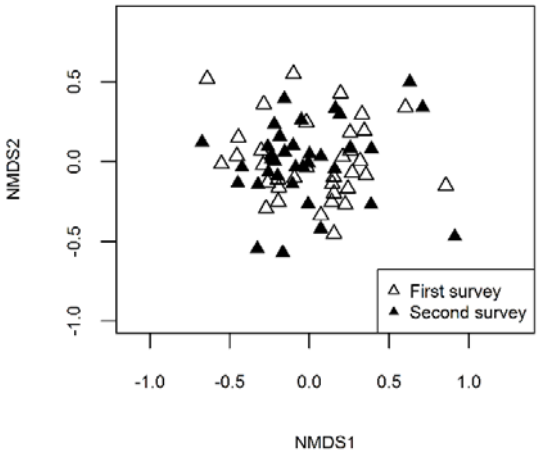


Fig. 2.4b NMDS ordination of species list data for the first and second surveys. Points represent grassland sites. Stress = 0.22.

The NMDS plots for management types show that there is some differentiation between the survey years (Figs. 2.5 and 2.6). In the meadow sites there is some separation along both axes for the quadrat data (Fig. 2.5a) with less difference between the two years in the species list data (Fig. 2.5b). In the grazed sites the differences between the two years are less distinct although two or three sites in each plot appear to have a different community composition than the majority of the grazed sites. Figs. 2.6a and 2.6b shows that change has taken place in sites which were managed as hay meadows at the time of the first survey and are now more intensively managed for silage or by permanent grazing. There is a less distinct pattern in the sites with little or no management (Figs. 2.6c and 2.6d).

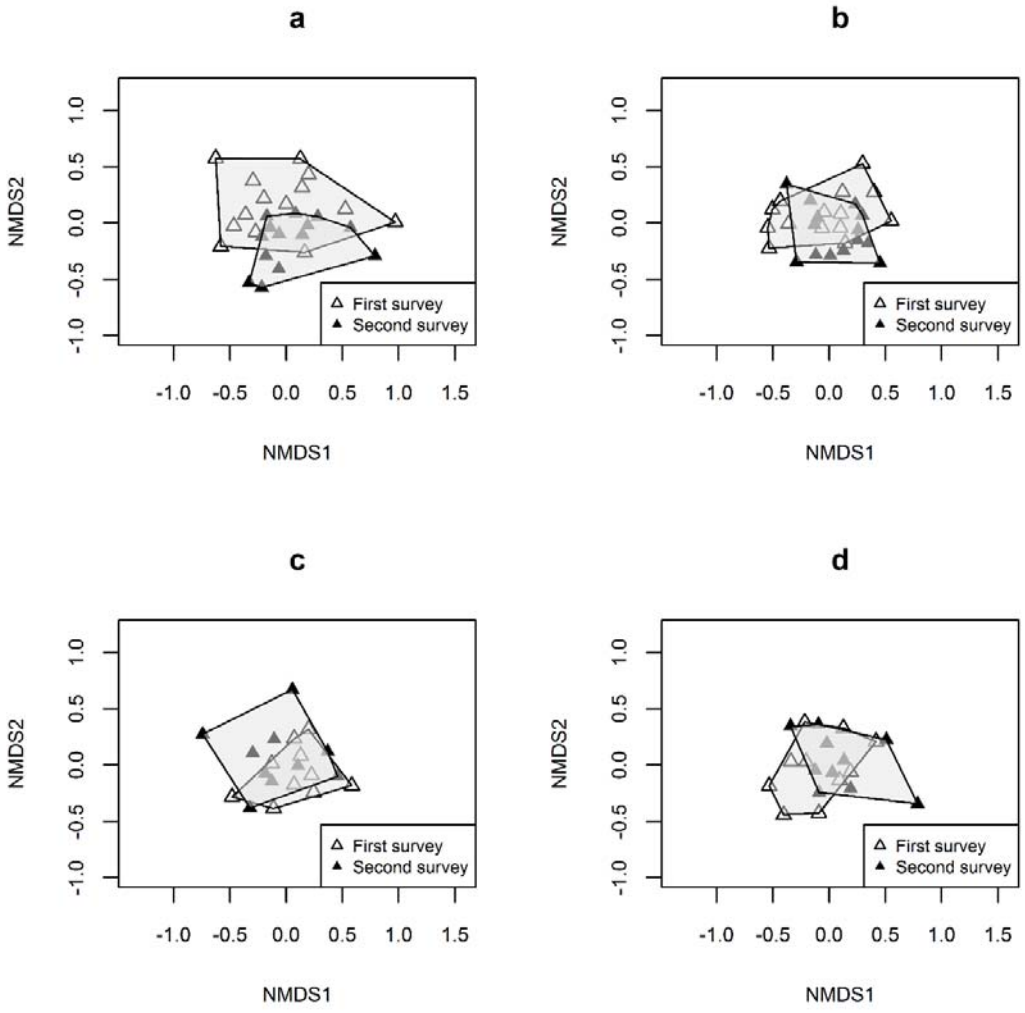


Fig. 2.5. NMDS ordinations of quadrat and species list survey data for the two survey years by management type. Plot **a** shows quadrat data for meadow sites (stress = 0.18), plot **b** shows species list data for meadow sites (stress = 0.17), plot **c** shows quadrat data for grazed sites (stress = 0.19) and plot **d** shows species list data for grazed sites (stress = 0.19).

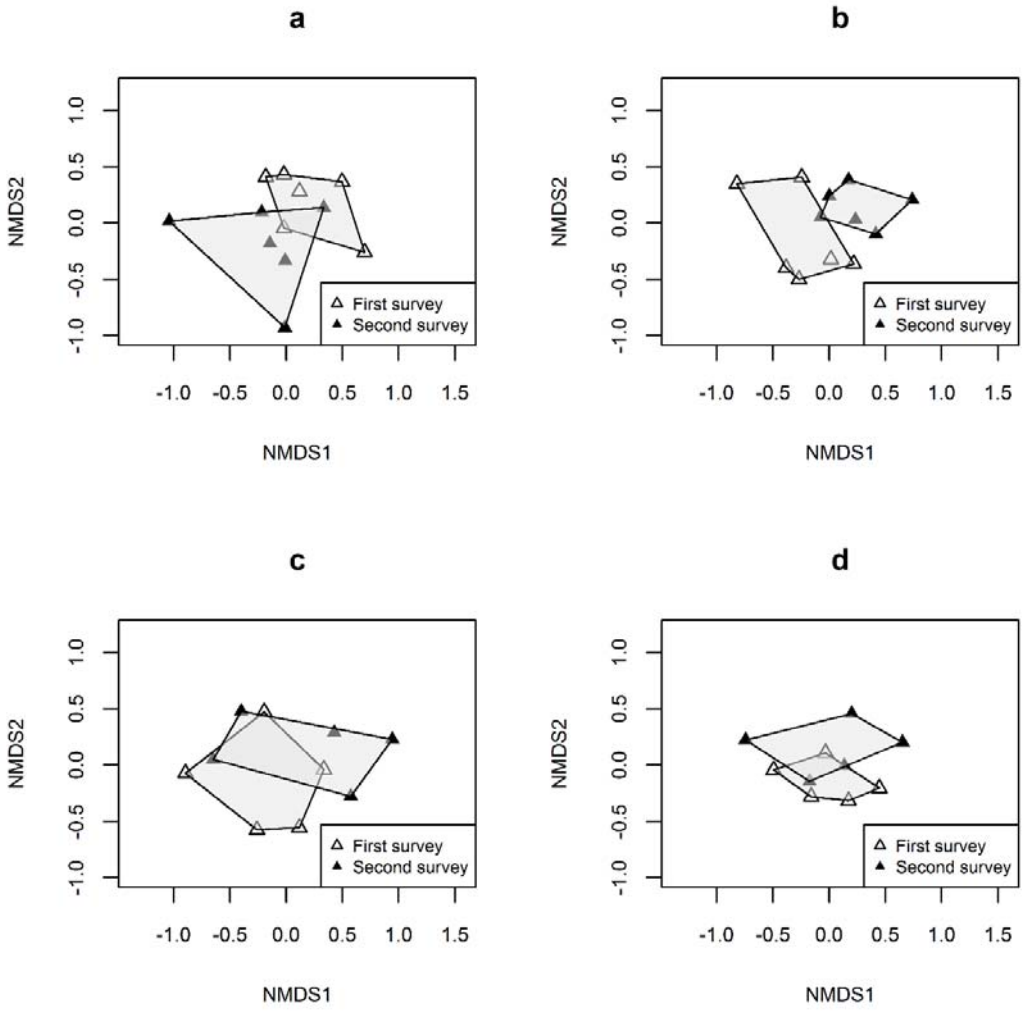


Fig. 2.6. NMDS ordinations of quadrat and species list survey data for the two survey years by management type. Plot **a** shows quadrat data for former meadow sites which are now more intensively managed (stress = 0.13), plot **b** shows species list data for intensively managed former meadow sites (stress = 0.13), plot **c** shows quadrat data for sites with little or no management (stress = 0.10) and plot **d** shows species list data for sites with little or no management (stress = 0.10).

The two quadrat and species list NMDS ordinations were not found to have a similar configuration. The Protest permutation test returned an *r* value of 0.27 which suggested that there was little correlation between the two ordinations. This result indicates that the two survey methods revealed contrasting results in terms of community composition.

More species had shown a decrease than an increase in terms of the number of site records (Tables 2.2 and 2.3). Table 2.2 shows the 25 species which showed the greatest decrease for both the quadrat and species list data (see Appendix 2 for a full species list). 11 of the 25 species appear in both the quadrat and species list data. Examples of species which are regarded as positive indicators for mesotrophic grassland were found in both sets of data (e.g. *Anemone nemorosa* and *Leontodon hispidus*). Some negative indicator species were also found to have decreased (e.g. *Dactylis glomerata)*. Species showing the most increases in site records are shown in Table 2.3. Fewer species had shown a substantial increase in site records, particularly in the quadrat data, but there were some examples of positive (eg. *Euphrasia* species) and negative indicator species (eg *Juncus effusus)*.

The analysis of increased and decreased species showed that there were higher EIV scores for light and moisture in the increased species for both quadrat and species list data (Table 2.4). For reaction (pH) there was a lower score in the increased quadrat species than the decreased but a higher score in the increased species list species. There was a similar pattern for fertility scores with a lower score for the increased quadrat species when compared to the most decreased species. The fertility score for the most increased species for the species list data appears to be substantially greater than that for the most decreased species although this was not tested statistically. Modal types for C-S-R signatures were different in the most increased species than the decreased species for both the quadrat data and the species list data with a shift away from the stress tolerator type.

Table 2.2 Decreases in number of records of species at grassland sites for quadrat survey data and species list data. The 25 most decreased species are shown. Species in bold are examples of positive indicators for UK mesotrophic grasslands and species with an asterisk* are examples of negative indicators (JNCC, 2004).

Quadrat survey data				**Species list survey data**			
Species	No of site records (1st survey)	No of site records (2nd survey)	Decrease in site frequency	Species	No of site records (1st survey)	No of site records (2nd survey)	Decrease in site frequency
Luzula campestris	20	6	-14	*Achillea ptarmica*	22	5	-17
Poa pratensis	14	2	-12	*Poa pratensis*	18	1	-17
Alchemilla glabra	**15**	**4**	**-11**	*Ficaria verna*	17	1	-16
Centaurea nigra	**27**	**16**	**-11**	*Luzula campestris*	25	12	-13
Achillea ptarmica	12	2	-10	*Achillea millefolium*	25	13	-12
*Dactylis glomerata**	20	10	-10	*Cardamine pratensis*	29	18	-11
*Phleum pratense**	17	7	-10	*Angelica sylvestris*	16	6	-10
Bellis perennis	18	9	-9	*Ajuga reptans*	13	4	-9
Bromus hordeaceus	13	4	-9	***Anemone nemorosa***	**9**	**0**	**-9**
Ficaria verna	12	3	-9	*Avenula pubescens*	14	5	-9
Conopodium majus	**19**	**11**	**-8**	***Alchemilla xanthochlora***	**8**	**0**	**-8**
Hypochaeris radicata	14	6	-8	*Cirsium vulgare**	8	0	-8
Leontodon hispidus	**13**	**7**	**-6**	*Festuca ovina*	16	8	-8
Plantago lanceolata	33	27	-6	***Leontodon hispidus***	**19**	**11**	**-8**
Prunella vulgaris	19	13	-6	*Ranunculus bulbosus*	9	1	-8

*Trifolium repens**	31	25	-6	*Bromus hordeaceus*	14	7	-7
Ajuga reptans	6	1	-5	***Centaurea nigra***	**30**	**23**	**-7**
Alchemilla xanthochlora	**5**	**0**	**-5**	***Conopodium majus***	**24**	**17**	**-7**
Anemone nemorosa	**5**	**0**	**-5**	*Phleum pratense**	21	14	-7
*Juncus inflexus**	6	1	-5	*Plantago major*	12	5	-7
Lathyrus pratensis	**22**	**17**	**-5**	*Vicia cracca*	21	14	-7
Ranunculus bulbosus	5	0	-5	*Agrostis capillaris*	34	28	-6
Sanguisorba officinalis	**21**	**16**	**-5**	*Cerastium glomeratum*	7	1	-6
Achillea millefolium	13	9	-4	*Heracleum sphondylium*	18	12	-6
Agrostis canina	4	0	-4	*Tussilago farfara*	8	2	-6

Table 2.3 Increases in number of records of species at grassland sites for quadrat survey data and species list data. The 15 most increased species are shown. Species in bold are examples of positive indicators for UK mesotrophic grasslands and species with an asterisk* are examples of negative indicators (JNCC, 2004).

Quadrat survey data				Species list survey data			
Species	No of site records (1st survey)	No of site records (2nd survey)	Gain in site frequency	Species	No of site records (1st survey)	No of site records (2nd survey)	Gain in site frequency
Ranunculus repens	20	31	11	*Alopecurus geniculatus*	8	19	11
Euphrasia species	**8**	**13**	**5**	***Galium palustre***	**6**	**17**	**11**
Galium palustre	**3**	**7**	**4**	*Juncus effusus**	15	25	10
Glyceria declinata	0	3	3	*Urtica dioica**	14	24	10
Lotus corniculatus	**13**	**16**	**3**	*Alopecurus pratensis*	14	23	9
Luzula multiflora	2	5	3	*Dactylorhiza fuchsii*	10	18	8
Myosotis discolor	5	8	3	*Juncus articulatus**	12	20	8
Alopecurus geniculatus	1	3	2	*Ranunculus repens*	28	35	7
*Juncus effusus**	5	7	2	*Myosotis discolor*	12	18	6
Trifolium dubium	1	3	2	*Poa trivialis*	26	32	6
Vicia cracca	8	10	2	***Euphrasia species***	**13**	**17**	**4**
Trifolium medium	0	1	1	*Galium aparine*	2	6	4
Triglochin palustre	0	1	1	*Glyceria declinata*	1	5	4
*Urtica dioica**	1	2	1	*Poa annua*	4	7	3
Vaccinium oxycoccos	20	31	11	*Alopecurus geniculatus*	15	18	3

Table 2.4 Mean Ellenberg Indicator Values (EIV) and C-S-R types for most increased and decreased species. Eb L = light; Eb F = moisture; Eb R = reaction; Eb N = fertility. C = competitor; S = stress tolerator; R = ruderal.

	Mean EIV				Modal C-S-R type
	Eb L	Eb F	Eb R	Eb N	
Most decreased species (quadrat data)	6.92	5.36	5.88	4.36	CSR
Most increased species (quadrat data)	7.07	6.50	5.36	4.29	CR
Most decreased species (species list data)	6.80	5.36	5.88	4.56	CSR
Most increased species (species list data)	6.93	6.43	6.29	6.21	CR

Discussion

Analysis of community composition

Taken as a whole the community composition of the 35 grassland sites had remained similar between the two survey years based on both the quadrat surveys and the species list surveys. This finding does not reflect the accounts of significant change in other grassland re-visitation studies (Bennie *et al.,* 2006; Bühler and Roth, 2011). The overall finding of limited change in the mesotrophic grasslands included in the present study may suggest that they are more resilient to change than other grassland habitats where the negative impacts of atmospheric nitrogen deposition and other sources of eutrophication on species richness or diversity have been greater (Stevens *et al*., 2010; Van den Berg *et al.,* 2011). Differences in the responses of grassland habitats to nitrogen deposition have been identified but the results are influenced by several factors including the baseline nutrient levels of the grasslands in the study (Maskell, *et al.,* 2010) and the varying effect of reduced or oxidised forms of nitrogen on the component species of acidic, calcareous or mesotrophic grassland communities (Van den Berg *et al.,* 2016).

Analysis by management type

Analyses of the community composition of the meadow sites in the first and second surveys indicated that there had been more change identified through the quadrat surveys than by the species list surveys. All of the meadow sites are subject to statutory protection and/or higher tier agri-environment schemes (AES) and have similar management regimes. It is possible that a particular aspect of this management regime is the reason for this change to the meadow community rather than a more widespread environmental impact which may have been more likely to have an impact on vegetation across the site. Another factor could be the effect of the isolation of populations of plants within the main meadow community since these sites are few in number and have a fragmented distribution. Detailed investigations of drivers of change are outside the scope of this study but more research into the significance of potential influences such as management, the fragmented distribution of sites, site location factors (eg altitude, aspect) as well as wider environmental factor such as nitrogen deposition would be valuable.

There is less clear evidence of change in the grazed sites although two sites from the second survey in the quadrat plot (Fig. 2.5c) show some separation from the others. One of these sites included plants associated with mire communities. The other site was being affected by encroachment of the woodland adjacent to it and supported woodland as well as grassland species when the second survey was undertaken. In the species list plot (Fig. 2.5d) some of the first survey sites show a degree of separation. Losses of species richness were recorded on these sites during the second survey which would account for differences in community composition. It was expected that overall there would be less similarity in the grazed sites due to the greater variation in terms of topography, hydrology and soil conditions, and in their management where livestock type, stocking density and timing of grazing could all influence the vegetation from site to site. However, this does not appear to be the case according to the data collected for this study. Unlike the meadows only three of the pasture sites are protected with the others either in lower tier AES schemes, which are less demanding in the management of grasslands for conservation (Natural England, 2013a; 2013b), or not part of any AES

agreement but this lack of a conservation framework for management does not appear to have led to significant change. The NMDS ordinations are valuable for comparing community composition across several sites but they are less useful in detecting fine scale changes which could be occurring within these sites. Hutson (1999) stressed the importance of scale in patterns of vegetation diversity and demonstrated that local conditions can influence regional diversity but such influences can be complex and are dependent on the scale of the study and the type of community.

It was expected that there would be significant change in the vegetation of the grasslands which had seen a change to a more intensive management regime since it is well documented that grasslands require regular low intensity management to maintain botanical diversity (Cuelmans *et al*., 2013; Klimek *et al.*, 2007; De Snoo *et al*., 2012). These sites do appear to have experienced the most change although there is not a complete separation of the two survey years. However, the small sample size of the changed sites means that the results have to be treated with some caution. Reference has already been made to the variations in site characteristics in pastures and the distinctiveness of individual sites was also a feature of the changed and unmanaged sites. For example one heavily-grazed former meadow site had retained many of the indicator species in the short sward whilst another with similar management had new records of some meadow indicators. Information on the dates for the changes in management was not available but research has shown that site management history and other small scale factors such as the current and past land use history of neighbouring sites as well as hydrological and soil conditions can all have a significant effect on current species diversity and composition (Gustavsson *et al*., 2007 Kalusová *et al.*, 2009, Reitalu *et al.*, 2009).

There is some evidence of change in the unmanaged sites although, again the small sample size must be taken into account. The lack of regular management appeared to have had an impact on species with a lower growth habit such as *Trifolium repens* and *Luzula campestris*. This is consistent with a study by Pavlů *et al.* (2011) which compared mown and unmanaged grasslands and reported similar

results where graminoids and forbs with a short growth habit occurred less frequently in unmanaged plots.

Findings from quadrat and species list surveys

In total more species were recorded in the species list surveys which was expected because the quadrat survey data is a sub-sample of the whole site. In the changed sites, for example, some species not found in the main sward had been retained on steeper banks at the edges of the sites. Some rare and uncommon species were picked up in the species list survey including *Primula farinosa, Platanthera chlorantha, Cirsium heterophyllum* and *Genista tinctoria* which have very few local records and are declining at the national level (Greenwood, 2012; Preston *et al.*, 2002). The comparison of the data resulting from the two survey methods showed that they had identified differences in terms of community composition. These differences can be explained by the fact that the species list survey required that all vegetation communities on the site were included. Features such as streams, ditches, areas close to a woodland boundary, gateways where there was evidence of eutrophication or more heavily trampled areas or small areas of acid or calcareous grassland which were not part of the quadrat survey were present on some sites. It is acknowledged that the effect of the sampling methods used should also be considered here. Surveyor bias and subjectivity will have some influence particularly in the compilation of the species lists so care is needed in the interpretation of the results. Ideally monitoring of long-term change should minimise sampling bias and error and the approach taken by Critchley & Poulton (1998) illustrates the value of precision and accounting for the optimum monitoring scale for different species. However, most revisitation studies aim to replicate the methods of the original survey so there is a trade-off between the value of the long-term data and the limitations imposed by the original survey design.

Species losses and gains

The changes in species records suggest a mixed picture in terms of the maintenance of the target plant communities of species rich mesotrophic sites. There were losses of some grassland species of conservation interest such as *Alchemilla glabra* which was only found in quadrats on 4 sites in the second survey (compared to 15 in the first), although losses recorded in the species list survey were less widespread (a decrease of 21 to 17 sites). Gains in positive grassland indicator species were also recorded (e.g. for *Euphrasia* species) but there were fewer gains than losses. There were losses and gains in site records for negative indicator species such as *Dactylis glomerata* which saw a substantial reduction in the quadrat survey and *Urtica dioica* which increased from 14 to 24 sites in the species list survey.

Some losses of positive indicators would be expected given the change to more intensive management in the former meadow sites but they may also reflect the impact of particular management prescriptions in sites which are being managed for conservation. For example, *Ranunculus repens* was recorded on all 35 sites and in most of the quadrats in the second survey. A study which investigated the control of *R. repens* and *Juncus* species (which also showed a large increase) found that early summer mowing dates were effective in reducing the abundance of *R. repens* whilst an autumn cut reduced *Juncus* species (Marriott *et al*., 2003). These cutting dates would not be permitted under AES management prescriptions for meadow sites.

The higher mean Ellenberg N score for the increased species in the species list data is mainly a result of increases in species like *Urtica dioica*, *Rumex obtusifolius* and *Galium aparine* which have Ellenberg N scores of 8 or 9. The Ellenberg N values in the increased species in the quadrat data were lower which could suggest that the species list scores were a result of localised increases of particular species. These species are also competitor species so their increases also influence the C-S-R scores. Ellenberg values and C-S-R strategies are useful but they may not take into account some

of the more subtle changes in the dynamics of these grassland communities, changes which may also be too fine scale for a regional analysis of community composition in all of the 35 sites in this study. Suding *et al.* (2005) found that whilst species richness always declined when soil nitrogen increased, there were varying responses among different plant traits and habitat types. Rare species and nitrogen fixing forbs were vulnerable to increases in fertility but so too were some perennials because of their conservative growth strategies in comparison to other more rapidly growing species which used the increased nitrogen more effectively. Conservation approaches which enhance rare species but also take account of the dynamics of different functional groups will require a greater understanding of these fine scale processes and how they relate to regional patterns of diversity, along with further long-term study to monitor their effectiveness.

Conclusion

The community composition of the 35 grassland sites had not seen a marked change at the regional level over the period of study. This is in contrast to the substantial changes noted in other re-visitation grassland studies. However important finer scale change was identified and grassland management had an influence on plant communities. Different survey methods provided contrasting information about the grassland sites and the combination of quadrat surveys and species lists can provide valuable information about key vegetation communities as well as other aspects of the site such as the presence of rare species. There were losses and gains of positive indicator species as well as changes in negative species but overall there were more losses than gains. This is a concern and more research is needed to understand why such losses are occurring particularly in sites which are protected and managed for conservation.

Chapter 3. Long–term hay meadow management maintains the target community despite local-scale species turnover

Introduction

Semi-natural grasslands support some of the most diverse vegetation in Europe but they have seen a marked decline since the middle of the 20th century (Eriksson *et al*., 2002; Hodgson *et al*., 2005b; Poschlod *et al*., 2005). Changing agricultural practices are the primary reason for this decline with intensification and abandonment resulting in reductions in biodiversity in grasslands although the pace of change in grassland habitats has differed from country to country (Baur *et al*., 2006; Dallimer *et al*., 2009; Otero *et al*., 2013). Semi-natural grasslands vary according to soil type, climatic conditions, altitude or local agricultural tradition (Küster and Keenleyside, 2009). This study focuses on mesotrophic meadows which are characterised by a low-input management regime with an annual hay cut and low intensity spring and autumn grazing (Crofts and Jefferson, 2007). At lower altitudes such meadows have been particularly vulnerable to agricultural intensification and cultivation because they were often located on sites which could be easily ploughed or drained. The resulting loss has meant that existing meadows are now highly fragmented (Eriksson and Cousins, 2014; Peterken, 2013).

Meadows which have a history of extensive management can support high levels of floristic diversity with over 30 species per square metre on the richest mesotrophic sites (Smith, 2010). The contribution of such habitats to international and national biodiversity has been acknowledged and many sites have been given statutory protection or included in agri-environment schemes (Jefferson, 2005). There is some concern, however, that these conservation measures are not maintaining the biodiversity of the sites they are designed to protect (Kleijn *et al*., 2006) and there is an increasing

demand for evidence to justify the resources required for managing agricultural land for conservation (Batáry *et al*., 2015).

The underlying mechanisms that enable diversity and variation in species composition in grassland ecosystems have been the subject of debate for some time (Yang *et al*., 2015). Factors which have been shown to influence diversity in grasslands include: levels of soil nutrients and moisture (Hejcman *et al*., 2014; Timmermann *et al*., 2015); the timing of mowing, and the timing and intensity of grazing (Smith *et al*., 1996); and the isolation of species-rich sites from sites supporting similar species (Krauss *et al*., 2004; Reitalu *et al*., 2009). The impacts of changes in nutrient levels, management regimes or fragmentation could be reflected in a reduction in species richness, a change in particular types of species or functional groups, or a loss of local distinctiveness in grassland vegetation (Čámská and Skálová, 2012; Homburger and Hofer, 2012; Wesche *et al*., 2012).

Obtaining evidence for the impact of change in grasslands requires a long-term approach because there will be a time lag in the response of vegetation to alterations in management or environmental factors (Helm *et al*., 2006). Such an approach is central to our understanding of the drivers of change in our most valued habitats, yet there is a limited availability of long-term ecological change data (Burt, 1994; Morecroft *et al*., 2009). Long-term experimental plots such as the Park Grass experiment (Silvertown *et al*., 2006) are an important source of ecological data which have been collected regularly and systematically, and which can be analysed with a degree of precision (Lindenmayer and Likens, 2012). There are clear advantages in this approach to the long term study of ecological change but there are also limitations in terms of the low numbers of experimental plot sites, and such experimental sites only cover small areas. (Hédl *et al*., 2017). Re-surveys of more extensive areas using historical data are a valuable addition to these studies since they increase our understanding of vegetation dynamics and ecological processes both spatially and temporally (Kapfer *et al*., 2017).

The value of re-visitation studies has been demonstrated in analyses of the response of vegetation communities to alterations in soil chemistry (McGovern *et al*., 2011), changes in fertiliser application

(Liira *et al*., 2012) or fragmentation of sites (Arponen *et al*., 2013) at various spatial scales. In previous re-visitation studies the focus has often been the substantial change in grassland community composition in regions where more intensive farming practices have replaced low input management (Krause and Culmsee, 2013; Prince *et al*., 2012; Wesche *et al*., 2012). Some studies of vegetation in meadows which have a long history of extensive management have investigated particular aspects of change such as the homogenisation of meadow vegetation (Bühler and Roth, 2011) and the loss of rare meadow species (Bradshaw, 2009). Only a small number of long-term studies have concentrated on meadows managed for both agricultural and conservation objectives. Critchley et al. (2007) considered the relationship between different aspects of management and vegetation change by looking at meadows in agri-environment schemes (AES) surveyed in 1987 and revisited in 2002. Since then there has been limited published evidence for the effectiveness of EU agri-environment schemes in meadows. The present study investigates long-term change in meadows which have all been managed for a hay crop but which were first surveyed prior to agri-environment management agreements or to the notification of statutory protection and thus addresses a significant gap in the literature.

In this study changes in plant communities in grasslands managed as meadows over a 25 year period were investigated. Data were obtained from sites first surveyed in the 1980s and repeat surveys were carried out in 2012. An analysis of overall community composition and species turnover was undertaken, and change in a particular suite of species which are representative of a mesotrophic meadow community was investigated. Change in soil conditions was assessed using Ellenberg Indicator Values since soil data was not collected in the original survey.

The study addressed the following questions:

1. What patterns of change can be identified in the community composition of meadow sites over 25 years?
2. Has a low-input management regime conserved species representative of a species rich hay meadow community?

3. What influence have soil conditions, site size and isolation had on the meadow community?
4. What are the implications for future conservation management?

Methods

Study area

The fourteen study sites are located in an area of approx. 17,000 ha in the Forest of Bowland in northern England at 53°58′N, 2°26′W (see Fig. 3.1). Climate and nitrogen deposition data are given in Appendix 3. Most of the meadows in the study are located at an altitude of 150-200m above sea level with a small number of meadows at lower levels. Table 3.1 lists site details.

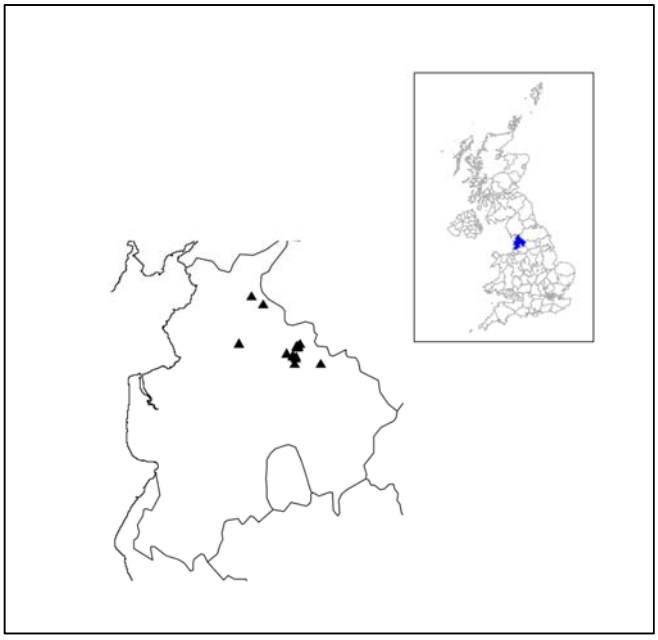


Fig. 3.1 Map of meadow site locations

Table 3.1 Details of meadow sites and information about area in hectares, nearest neighbour distance in kilometres, altitude in metres above sea level (m a.s.l.) and site designation. SAC = Special Area of Conservation (European designation), SSSI = Site of Special Scientific Interest (UK designation)

Site ID	Site area (ha)	Distance to nearest meadow site(km)	Altitude (m a.s.l.)	Statutory Designation
BG	5.47	0.01	180	SSSI
BG2	2.2	0.01	180	None
BG3	3.1	0.13	180	None
BS (3 fields)	7.65	0.01	150	SAC/SSSI
BS1	2.3	0.01	180	None
CB	0.54	3.49	60	SSSI
DH	0.4	0.78	190	None
FH	1.63	3.49	105	SSSI
FHM	3.33	0.46	210	SSSI
LCM	5.26	0.46	190	SAC/SSSI
MM (2 fields)	9.09	1.29	155	SAC/SSSI
NI	2.09	6.13	125	SSSI
SM	3.63	1.20	200	None
TB (5 fields)	11.87	11.59	155-180	SSSI

Three of the meadow sites form part of the North Pennine Dales Meadows Special Area of Conservation (SAC) and nine meadows have the national statutory designation of Sites of Special Scientific Interest (SSSI). The meadows are described as belonging to the Triseto-Polygonion alliance or are associated with alliances within the Molinio-Arrhenatheretea order (Rodwell *et al.*, 2007). Within the UK National Vegetation Classification they support MG3 *Anthoxanthum odoratum-Geranium Sylvaticum*, MG4 *Alopecurus pratensis-Sanguisorba officinalis* and MG5 *Cynosurus cristatus-Centaurea nigra* communities (Rodwell, 1992).

All of the sites are protected and/or in an agri-environment scheme so their management involves a late hay cut (after 15 July for AES sites) followed by field drying of the hay for 3-4 days and baling. Aftermath grazing is carried out in the late summer/early autumn and the meadows are grazed in the spring until they are 'shut up' to allow the hay crop to grow. Livestock are removed for a period of time before the hay cut (usually a minimum of 8 weeks) and a limited amount of farmyard manure may be spread on the sites. (Natural England 2010, 2017b, 2017c). There will be minor variations in the management regime because of weather conditions and the type of livestock on each farm.

Field survey and data collection

Surveys of semi-natural grassland were carried out in England in the 1980s and 1990s and the results were compiled into the Grassland Inventory for England (Blackstock *et al*., 1999; Jefferson *et al*., 1997). The detailed survey records for the county of Lancashire survive in their original form, and it is these records which were the baseline for this study. From these inventory records 14 sites were identified in the Bowland region which had been consistently managed as meadows since the 1980s. In the original surveys the sites had been described as species-rich meadow communities and references were made to the classifications MG3, MG4 or MG5 grassland although the surveys took place before the publication of the National Vegetation Classification for grasslands in 1992 (Rodwell, 1992).

The guidance for the original surveys (hereafter the 'first survey') stated that $1m^2$ quadrats should be placed randomly within a stand of vegetation judged to be representative of the meadow community (Smith *et al*., 1985). The numbers of quadrats varied according to the size of the site but in total 55 quadrat surveys were recorded across the 14 sites. In these quadrats all vascular plants were recorded using the Domin scale of cover-abundance. The survey record cards also included sketch maps which showed the locations of the quadrats in each field (Smith *et al*., 1985).

Copies of the original record cards were used to locate the approximate positions of the quadrats and the surveys were carried out using the original methodology. Most of the sites consist of individual fields and the larger sites are sub-divided into field units so it was possible to place the quadrats within a few metres of the original locations. In addition the meadow sites have a relatively uniform sward so choosing an area which was representative of the whole stand was straightforward. Tests for plot relocation accuracy were applied to the quadrats from a random selection of 4 of the meadow sites following the method developed by Ross *et al*. (2010). Wilcoxon rank sum tests showed that there was significantly greater dissimilarity between the first and repeat survey quadrats compared with the dissimilarity between the among survey quadrats ($P < 0.001$). The repeat surveys (the 'second survey') were carried out in the summer of 2012 as closely as possible to the original survey dates and were completed by mid-July to ensure that they were completed before the hay cut. All of the 55 quadrats from the first survey were re-surveyed and the locations of the second survey quadrats were recorded using a handheld GPS.

Soil data were not collected during the first survey so changes in soil conditions were compared using Ellenberg Indicator Values for the British flora (Hill *et al*., 1999). Ellenberg Indicators give a proxy value for soil conditions based on scores awarded to vascular plants. Cover weighted and non-weighted Ellenberg scores were compared across the sites but there was little difference in the results so non-weighted scores were used in the analysis. The size of the sites were calculated in hectares (ha) and isolation of sites was calculated by measuring the straight line distance in km between one meadow and the closest neighbouring meadow (nearest neighbour distance).

Percentage cover of key meadow species was recorded as a subset of the overall data set. Species used as both positive and negative indicator species for meadow vegetation in condition assessments of UK species rich grasslands were included in this subset (JNCC 2004) along with species used in a study by Kirkham *et al*. (2014) which compared change in an upland and lowland meadow. In the UK context the Bowland meadows are mainly upland sites but some are found at lower levels so

these lists of species were found to be particularly relevant to the present study. A full list of indicator species is found in Appendix 4.

Data analysis

All statistical analyses were carried out in R version 3.1.2 (R Development Core Team, 2014). Tests for normality of distribution and homogeneity of variance were carried out where appropriate. Domin scores in both surveys were converted to a percentage cover value using the Domin 2.6 transformation (Currall, 1987) to avoid over emphasising rare species.

To analyse differences in community composition between the two surveys redundancy analysis (RDA) was carried out using the vegan package in R (Oksanen *et al*., 2013). The data were transformed using the Hellinger transformation (Legendre and Gallagher, 2001) and the RDA was performed with survey year as the explanatory variable and site as a covariable.

A permutation test was used to indicate the level of statistical significance of the final model (minimum permutations = 1000). The eigenvectors (species scores) returned by the model were used to establish which species had contributed most to the variance in species composition. The species which were the most associated with each of the two survey years based on the magnitude of the eigenvectors were extracted from the model output and an analysis was made of which species had increased or decreased in terms of frequency of records on each site between the two survey years. To investigate change in site species richness the mean number of species per site was calculated and the difference between the two survey years was tested using a paired *t*-test.

To investigate patterns in the diversity of the meadow community vegetation a test for multivariate homogeneity of group dispersions was carried out using the betadisper function in vegan followed by a permutation test for significance (Anderson *et al*., 2006; Oksanen *et al*., 2013). This function

involves the calculation of distances between the principal co-ordinates of dissimilarity coefficients to group centroids. In this case the Bray Curtis dissimilarity index was used because it takes account of relative abundances and has the necessary properties for the analysis of betadiversity (Legendre and De Cácares, 2013). The groups were the two survey years.

Analyses of changes in soil conditions were carried out by using permutation tests on unweighted mean Ellenberg indicator values for soil moisture (Ellenberg F), pH (Ellenberg R) and fertility (Ellenberg N). The permutation tests followed the method developed by Zelený and Schaffers (2012) which was designed to overcome the bias associated with analysis of Ellenberg indicators when they are compared using parametric tests or used as explanatory variables in constrained ordination.

Differences in percent cover of positive indicator species by site was tested by a Wilcoxon signed rank test to account for a non-normal distribution. Correlations between positive indicator species cover and site characteristics (size, nearest neighbour distance and altitude) were tested using Spearman rank correlation tests.

Results

95 species were recorded across the 14 meadow sites in the first survey whereas 79 species were found in the second survey. Site richness was significantly lower in the second survey than it was in the first survey.

Results of the RDA showed that there was a significant difference (P = 0.003) between the community composition of the meadow sites in the first survey compared with that of the second survey (Fig. 3.2). Survey year accounted for 11.26% of the variance and the adjusted R^2 value was 0.078.

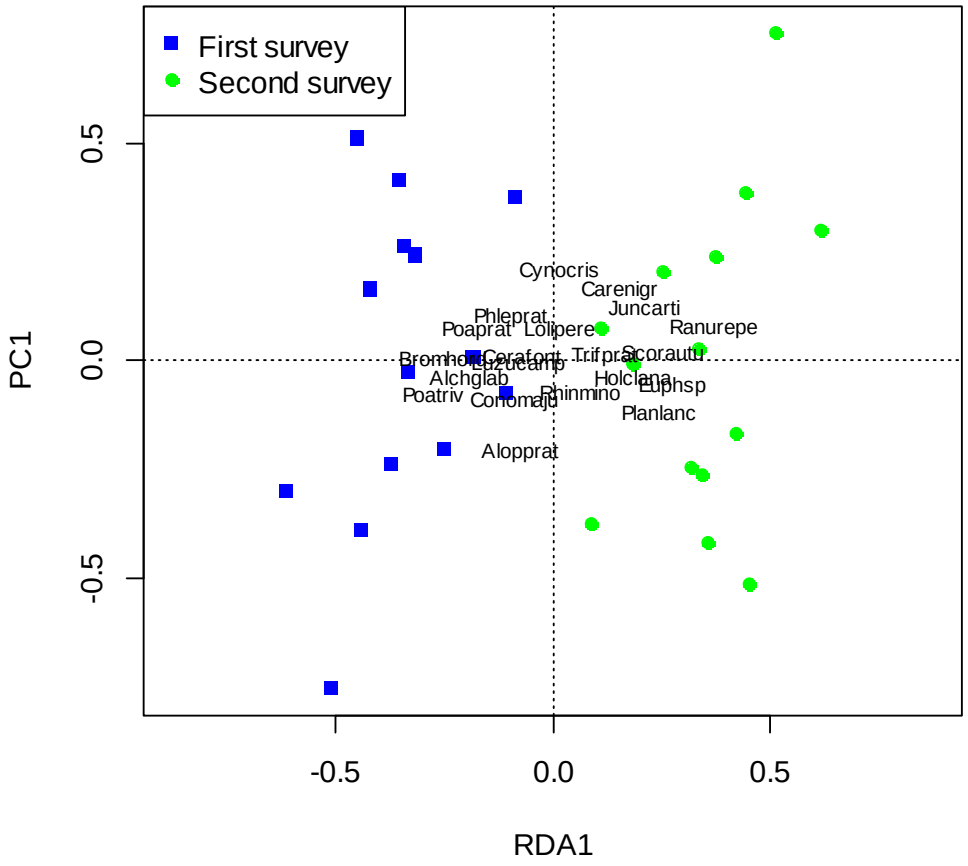


Fig. 3.2 RDA ordination plot of meadow sites constrained by year. Squares symbols show sites in first and second survey. Ten species most associated with first and second survey are shown. Alchglab = *Alchemilla glabra;* Alopprat = *Alopecurus pratensis;* Bromhord = *Bromus hordeaceus;* Carenigr = *Carex nigra;* Cerafont = *Cerastium fontanum;* Conumaju = *Conopodium majus;* Cynocris = *Cynosurus cristatus;* Euphsp = *Euphrasia* species; Holclana = *Holcus lanatus;* Juncarti = *Juncus articulatus;* Lolipere = *Lolium perenne;* Luzucamp = *Luzula campestris;* Phleprat = *Phleum pratense;* Planlanc = *Plantago lanceolate;* Poaprat = *Poa pratensis;* Poatriv = *Poa trivialis;* Ranurepe = *Ranunculus repens;* Rhinmino = *Rhinanthus minor;* Scorautu = *Scorzoneroides autumnalis;* Triprat = *Trifolium pratense*

The species scores for the first axis were extracted from the RDA output and were ranked according to their magnitude. The 10 species with the lowest negative eigenvector scores (most associated with the first survey) and the 10 species with the highest negative scores (most associated with the second

survey) are shown in Table 3.2. Table 3.2 shows whether these species increased or decreased in terms of the frequency of sites in which they were recorded. Positive and negative meadow indicator species are also identified. Some positive indicator decreased such as *Conopodium majus* and *Alchemilla glabra* whilst others increased such as *Euphrasia species*, *Scorzoneroides autumnalis and Rhinanthus minor*. There were also decreases in some negative indicator species, eg *Bromus hordeaceus, Phleum pratense* and *Lolium perenne*, whilst others such as *Ranunculus repens, Juncus articulatus* and *Holcus lanatus* saw an increase. Decreases also occurred in common grasses such as *Poa trivialis* which is not considered to be an indicator species and there were increases in widespread grassland species such as *Plantago lanceolata* and *Trifolium pratense*.

Table 3.2 Species with 10 lowest negative and 10 highest positive eigenvector scores in RDA. Rank of scores is listed along with frequency of records for each species in sites in the first and second surveys. Positive species are identified by * and negative species by #. Sources: JNCC (2004); Kirkham et al (2014).

	Rank of RDA score	Site records 1st survey	Site records 2nd survey	Change in site frequency
Species most associated with first survey (negative eigenvector scores)				
Poa trivialis	105	11	9	-2
#Bromus hordeaceus	104	9	4	-5
#Phleum pratense	103	9	4	-5
**Conopodium majus*	102	9	6	-3
Poa pratensis	101	4	1	-3
**Alchemilla glabra*	100	8	3	-5
Luzula campestris	99	6	2	-4
Alopecurus pratensis	98	8	6	-2
#Lolium perenne	97	10	9	-1
Cerastium fontanum	96	12	10	-2
Species most associated with second survey (positive eigenvector scores)				
#Ranunculus repens	1	5	14	+9
**Euphrasia spp*	2	5	11	+6
Plantago lanceolata	3	13	14	+1
**Scorzoneroides autumnalis*	4	5	10	+5
#Juncus articulatus	5	2	5	+3
Trifolium pratense	6	9	12	+3
Cynosurus cristatus	7	11	14	+3
#Holcus lanatus	8	13	14	+1
**Rhinanthus minor*	9	13	14	+1
Carex nigra	10	3	3	0

The analysis of homogeneity of community composition in the two sites revealed that mean distances between centroids had decreased from 0.42 to 0.36 (Fig. 3.3) and the permutation test revealed that the differences between the distances were significant (P = 0.04). This result indicates that the vegetation has become more homogenous over the survey period.

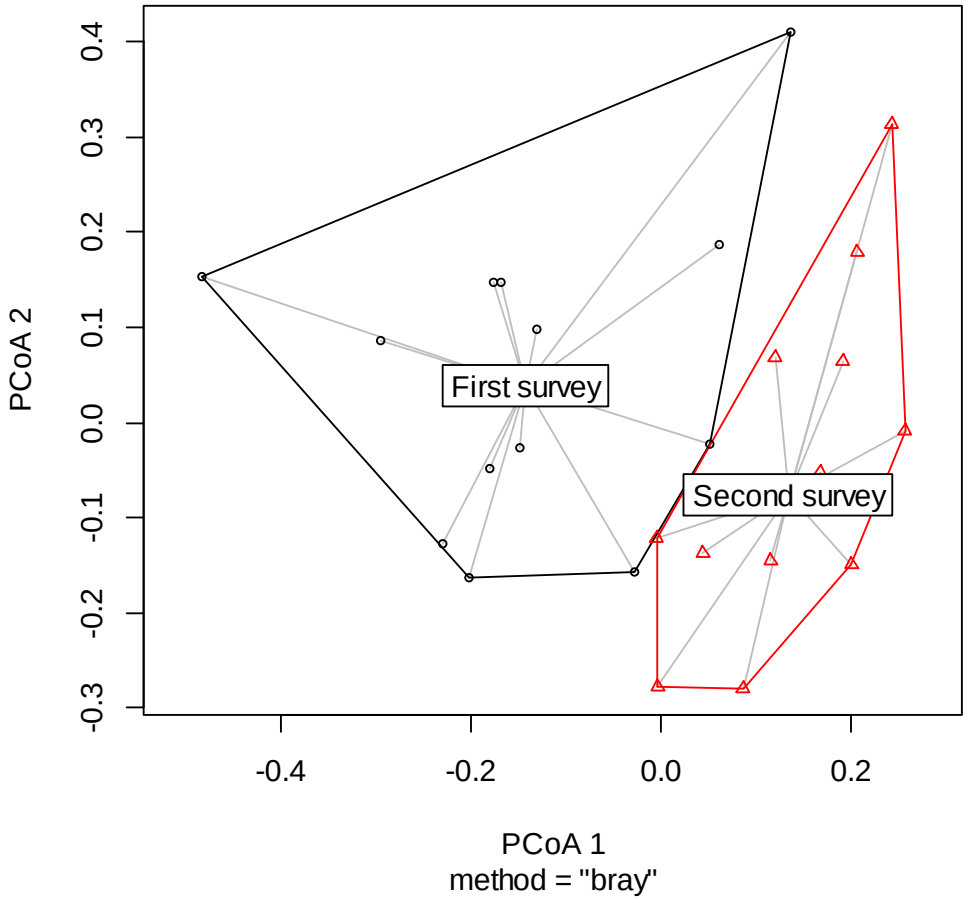


Fig. 3.3 PCoA plot showing analysis of homogeneity of multivariate dispersions using betadisper function with Bray Curtis distance matrix. The mean distances to the centroid was 0.42 for first survey and 0.36 for the second survey.

There was no significant difference in any of the mean Ellenberg indicator values between the two survey years (Table 3.3) suggesting that differences in soil conditions were minimal. Site richness was significantly lower in the second survey than it was in the first survey (Table 3.3).

Table 3.3 Changes in Ellenberg F, R and N indicators, percent cover of positive and negative meadows species, comparison of site species richness by year and results of correlations between cover of positive species and site size, nearest neighbour distance and altitude between the first and second survey. *P* values significant at the 0.05 level are identified by* and *P* values of < 0.01 are identified by **. Non-significant results are identified by (NS).

	Test	**Result**	
Ellenberg F	Modified permutation test		F = 0.96, *P* = 0.307(NS)
Ellenberg R	Modified permutation test		F = 3.05, *P* = 0.066(NS)
Ellenberg N	Modified permutation test		F = 2.26, *P* = 0.111(NS)
% cover of positive species	Wilcoxon signed rank		V = 130, *P* = 0.368(NS)
% cover of negative species	Wilcoxon signed rank		V = 110, *P* = 0.032*
	Test	**First survey**	**Second survey**
Difference in site species richness	Paired *t*-Test		*t* = 3.32, *P* = (0.005)**
Site species richness	Mean (standard error)	31.71(2.78)	27.64(2.36)
Correlation: cover of positive meadow species and site size	Spearman rank	Rho = 0.08, *P* = 0.784(NS)	Rho = 0.17, *P* = 0.562(NS)
Correlation: cover of positive meadow species and nearest neighbour distance	Spearman rank	Rho = 0.31, *P* = 0.278(NS)	Rho = -0.26, *P* = 0.376(NS)
Correlation: cover of positive meadow species and altitude	Spearman rank	Rho = -0.47, *P* = 0.092(NS)	Rho = -0.16, *P* = 0.576(NS)

An analysis of the change in the percentage cover of positive meadow species between the first and second surveys did not reveal a significant difference but there was a significant decline in the percentage cover of negative species (see Table 3.3). There were no significant correlations between positive indicator species and the site characteristics of size, nearest neighbour distance and altitude. However plots of change in percentage cover of positive meadow species by location (Fig 3.4a and 3.4b) showed that cover had decreased at the more isolated sites whilst sites which were closer together with had seen increases.

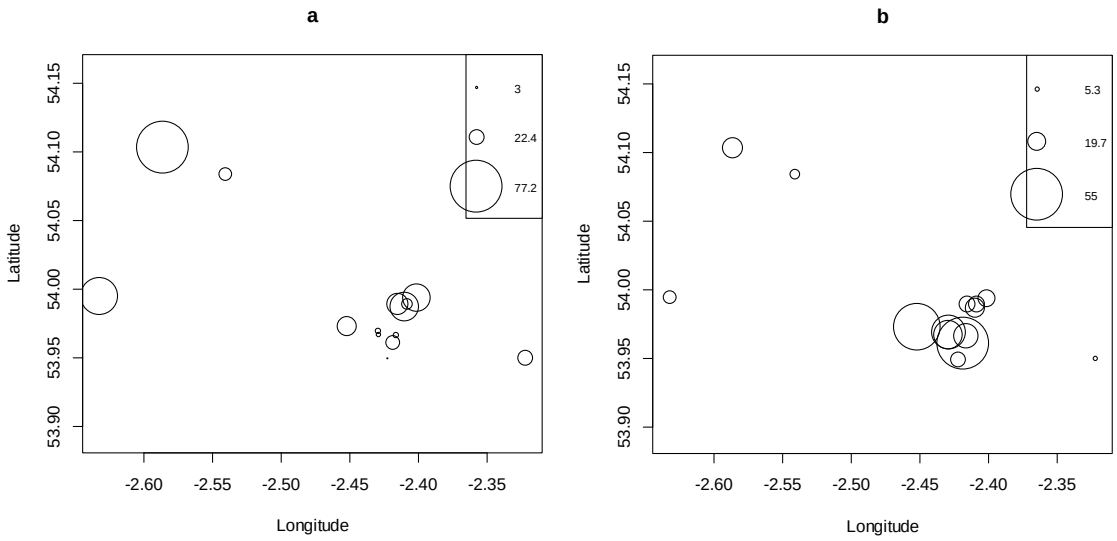


Fig. 3.4 Plot of cover of positive indicator species in the second survey by location. The larger the circle the higher the percent cover value. Legend shows largest and smallest cover values and median values. Plot a shows the first survey and plot b shows the second survey.

Discussion

Patterns of change in community composition

The analysis of meadow sites between the two survey years has shown that there has been a significant change in community composition, and that this change has been brought about by a reduction in negative meadow indicator species and by turnover in some of the key positive species which are representative of the meadow community. The cover of positive meadow species as a whole appears to have been maintained but there has been an increase in homogeneity and the patterns of change were not consistent across all the sites in the study.

Reduction in negative species

Thirteen of the fourteen meadows sites are managed through an agri-environment scheme (AES) and nine are protected through a statutory designation. These measures allow limited amounts of farmyard manure to be applied but prohibit the use of inorganic fertilisers. There is substantial evidence to show that increased soil fertility encourages the growth of competitive grasses, particularly on sites with a history of low input management (Kirkham *et al*., 2014; Mountford *et al*., 1993; Smith *et al*., 2003) so it would be expected that limiting fertility would restrict negative species such as *Lolium perenne* and *Phleum pratense* which were shown to decrease in the study area.

It is also notable that two hemi parasitic herbs *Euphrasia* species and *Rhinanthus minor* have shown an increase in the frequency of sites on which they were recorded. The ability of hemi parasitic species to suppress competitive grasses has been demonstrated in studies of meadow restoration (Bullock and Pywell, 2005) so it is possible that these two species have played a role in the reduction of negative species. Overall there was a reduction in cover of negative indicator species (Table 3.3) but there were increases and decreases in the frequency of individual species indicating that the overall pattern is not reflecting the detailed change at the individual site and species level.

Turnover in positive meadow species

The analysis of percentage cover of positive meadow species across the study area showed that there was no significant difference between the two survey years but it is clear that there are 'winners' and 'losers' among the positive species. For example *Alchemilla glabra***,** which has seen a decline, is a constant species in the upland hay meadow community (MG3) (Rodwell, 1992). *A. glabra* is declining nationally particularly at the southern edge of its range in northern England and parts of Wales (Preston *et al*., 2002). Studies of upland hay meadows in northern England have linked change in key indicator species primarily to management, habitat quality and fragmentation (Bradshaw, 2009; Critchley *et al*., 2007; Pacha and Petit, 2008) but higher temperatures resulting from climate change may become more of a concern for the northern montane species in the future so monitoring of such species is important for conservation.

A study of hay meadows by Valkó *et al*., (2012) found that mowing was needed to limit graminoid biomass but that some species are supressed by mowing. These species include *Achillea ptarmica* which declined in frequency but was not one of the 20 most influential species in the RDA. It was suggested that greater diversity in the meadow sward could be achieved by introducing variations in mowing regimes, eg, by including fallow years or leaving unmown strips. Whilst this approach may not be as attractive in terms of agricultural productivity it may reflect more closely the pattern of meadow management prior to the introduction of AES. Farmers may have chosen to mow earlier or later depending on levels of spring growth, weather conditions or even availability of labour (Eriksson *et al*., 2015). The use of a standardised cutting date applied to whole regions of northern England has been linked with a widespread increase in annual species such as *Euphrasia* species. The regular pattern of later cutting is thought to allow annuals to set seed every year whereas prior to the introduction of AES dates would have varied with some years being less favourable for annuals (O'Reilly, 2010; Starr-Keddle, 2014).

There has also been concern that the use of a standardised approach could be linked to the loss of local distinctiveness or increased homogenisation in species rich grasslands (Homburger and Hofer, 2012). Bühler and Roth (2011) found that taxonomic homogenisation was a result of an increase in more common species. An example in this study could be *Scorzoneroides autumnalis* or *Trifolium pratense* which are common grassland 'generalists'. It is likely that a longer time period is needed to assess such changes particularly in grasslands where most of the species are perennials (Bühler and Roth, 2011). The need to balance regionally applied management prescriptions, which appear to maintain the overall suite of meadow species in the study area, with the long-term impact on floristic homogenisation and local distinctiveness merits further investigation.

Effect of fragmentation of grassland sites

There were no significant relationships between site size, nearest neighbour distance and altitude with cover of positive meadow species. However, the pattern of losses and gains in percent cover by site shown in Fig 3.4 could indicate that change is taking place at the site level which is not yet statistically significant but may become increasingly important from an ecological point of view.

The loss of specialist plant species with increasing fragmentation of semi-natural grasslands has been reported in other studies (Brückmann *et al*., 2010; Pacha and Petit, 2008). There is evidence that smaller populations of grassland specialists may be more vulnerable to fragmentation but the time taken before the effects of isolation become evident will vary depending on population sizes, time since fragmentation occurred and other factors (Kuussaari *et al*., 2009). The impacts of isolation in grasslands on genetic diversity and the subsequent reduction in plant fitness have been highlighted along with the need to account for slow response times to habitat fragmentation (Takkis *et al*., 2013). A precautionary grassland management approach which takes into account the connectivity of protected sites and other grasslands of conservation value has been recommended as the way to address the effects of isolation of grassland populations (Arponen *et al*., 2013).

Soil conditions

The influence of Ellenberg F values were not found to be significant but the increases of (*Ranunculus repens* and in *Juncus articulatus*) could be indicative of a shift towards conditions where drainage is impeded (Lynn and Waldren, 2003; Rodwell, 1992). The impact on soil compaction and alterations to soil hydrology resulting from the use of heavier farm machinery and from trampling by cattle has been well documented (Hamza and Anderson, 2005; Kurz *et al*., 2006). In addition there were no options in the AES for farmers to repair or replace collapsed field drains, and the installation of new drainage systems is not permitted on protected grassland sites (Natural England 2017b). This is likely to have resulted in field drain maintenance being neglected. However, the widespread increase in *R. repens* from 25% to 84% of the quadrats indicates a distribution throughout the sward. It would be expected that localised increases in moisture associated with damaged field drains would have shown a more patchy distribution.

The nitrogen deposition value for the area (27.02 Kg N/ha/year) is close to the upper limit of the critical load for low and medium altitude hay meadows which is given as 20-30 Kg N/ha/year (Appendix 3) but analysis of mean Ellenberg N values did not show a significant increase from the first to the second survey and the values for both surveys were within the target range used by Smith *et al*. (2003) in their study of upland meadow restoration i.e. approximately 4.3-4.7 for mean Ellenberg N values. Findings from other long term studies grassland studies have shown that the impact of nitrogen deposition on grassland vegetation appears to vary according to grassland type with notable negative impacts on species richness in acid grasslands (Stevens *et al*. 2010). Mesotrophic grasslands are less well studied but Van den Berg *et al*. (2016) reported a negative relationship between nitrogen deposition and species richness in mesotrophic grasslands as well as in acid grasslands, and found that there was a positive effect on species richness in calcareous grasslands.

Values for Ellenberg R were also similar for both surveys. Acid deposition for the area did not exceed critical loads during the period of the second survey (APIS, 2017) although the first survey would have been undertaken at a time when acid deposition levels are likely to have been higher than at present (RoTAP, 2011).

It should be noted that Ellenberg values are a proxy measure and can differ from analyses of soil chemistry (McGovern *et al*., 2011) but they do provide a useful indication of change where soil data is not available for comparison. In the present study it appears that hay meadow management has maintained soil fertility, pH and moisture levels overall although the effects of nitrogen deposition and potential impacts of impeded drainage should be monitored to inform future management approaches.

Conclusions

There has been a significant change in the community composition of hay meadow vegetation in the study area but hay meadow management has maintained the overall cover of positive meadow species and reduced the cover of negative species. There have been losses of particular species which are key constituents of the hay meadow communities and the meadow vegetation has become more homogenised. The measurement of success of the conservation of hay meadows should take account of the impacts on key species and on individual sites, and consideration should be given to a more targeted approach to management prescriptions. There was no significant change in Ellenberg values, and more research is required to explore the reasons for species change and to establish whether the fragmented distribution of the meadows is affecting their long-term viability, findings which have also been identified in studies across Europe.

Chapter 4. The impact of fragmentation on the genetic diversity and population structure of a key hay meadow species, *Rhinanthus minor* L.

Introduction

The importance of the species rich hay meadow habitats which results from their botanical diversity has been discussed in Chapter 3. This study has highlighted increases and decreases of some key meadow species and has proposed possible reasons for these changes including particular aspects of the management regime, environmental change or the effect of the fragmented distribution of the sites. The details of hay meadow management and the influence of environmental change such as aerial nitrogen deposition on vegetation composition have been widely studied (see, for example Smith *et al*., 1996; Stevens *et al*., 2010) but the impact of fragmentation on hay meadow plant populations has received little attention. The results described in Chapter 3 showed that there was some indication of losses of key meadow species at the more isolated meadow sites in the Bowland region, and this chapter investigates whether the fragmented distribution of meadow sites is having, or is likely to have in the future, a negative impact on the viability of plant populations by examining genetic variation in the Bowland meadows.

Fragmentation, and the accompanying loss of genetic diversity, has been described as one of the primary threats to biodiversity (Gibbs, 2001; Hanski, 2005; Hanski, 2011), and the study of connectivity between populations has been recognised as a major element of conservation science (Kool and Moilanen, 2013). Studies of the impact of fragmentation have taken a number of approaches including the investigation of relationships between patch size and species richness, analysis of the influence of past landscapes and habitats, and different aspects of functional

connectivity (Auffret *et al*., 2015; Krauss *et al*., 2004; Purschke *et al*., 2014). These approaches can be enhanced by the use of genetic tools to examine connectivity between populations (Baguette *et al*., 2013) whilst estimates of genetic diversity within isolated populations can provide further insights into the impact of fragmentation (Leimu *et al*., 2006).

Reductions in genetic variability within populations and genetic divergence between populations would be expected to result from the fragmentation of habitats (Young *et al*., 1996). Smaller and more isolated populations are likely to be more susceptible to genetic drift, higher levels of inbreeding and reduced gene flow between populations (Ellstrand and Elam, 1993). Studies of plants which were formerly widespread in their distribution but are now found in small, fragmented populations have confirmed low levels of genetic diversity (Crichton *et al*., 2016; Jacquemyn *et al*., 2010). In contrast, naturally isolated populations, such as those found in mountainous regions, have shown high intra-population diversity albeit with high levels of population differentiation (Ægisdóttir, *et al*. 2009). Moreover, the impact of fragmentation is not only felt in populations of rare plants. More common species have also been subject to reduced genetic diversity where they are found in small populations which were historically part of larger or more connected populations (Galeuchet *et al*., 2005 Honnay and Jacquemyn, 2007; Van Rossum *et al*., 2004). There is empirical evidence for population differentiation among fragmented populations (Heinicke *et al*., 2016; Vandepitte *et al*., 2013) although levels of differentiation were lower than expected in some fragmented populations (Gómez-Fernández *et al*., 2016) and varied according to species even within the same habitat (Durka et *al*., 2017).

The precise effects of fragmentation of genetic variability and plant fitness are not fully understood (Heinken and Weber, 2013; Picó and Van Groenendael, 2007). Reduced mean fitness has been shown to correlate with small population size in a study by Oakley and Winn (2012) but the effects of population size on inbreeding depression are less straightforward (Angeloni *et al*, 2013). There is

agreement in the literature that the impact of fragmentation is complex, particularly in plant populations and factors such as mating systems, longevity, time since fragmentation occurred, interaction with pollinators, seed dispersal and storage ability could all be important (Picó and Van Groenendael, 2007; Young *et al*., 1996). For example, Leimu *et al*., (2006) found that fitness increased with genetic variation in self-incompatible species but this relationship did not apply in self-compatible species and that the negative impact of small population size was equally important in short- and long-lived species. Likewise Honnay and Jacquemyn (2007) noted that the positive correlation between population size and genetic diversity was more pronounced in self-incompatible but mainly outcrossing species and obligate outcrossing species than it was in self-compatible species but, interestingly, that this effect was equally important in common species as it was in rare species. Historical patterns of habitat connectivity can be an important influence on current levels of genetic diversity although this may be more applicable to long-lived perennial species (Münzbergová *et al*., 2012: Reisch *et al*., 2017) and changes in pollinator behaviour as a result of fragmentation can also have a negative effect on genetic diversity (Ellstrand and Elam, 1993: Kwak *et al*., 1998).

Conservation of species rich grassland sites including hay meadows has been mainly focused on maintaining or enhancing species diversity in individual sites (Gaston et al, 2006). More recently there has been a recognition that conservation should take a landscape-scale approach which considers habitat connectivity and thus addresses fragmentation (Donald and Evans, 2006). An understanding of the current levels of genetic variability and the extent of gene flow in fragmented habitats is key to the long-term success of this approach since it provides an indication of the viability of ecological connections between habitats. Other studies have provided valuable information about gene flow between grassland plants in different circumstances: Jacquemyn *et al*. (2010) investigated a rare plant found in fragmented grassland patches which had limited gene flow whilst Aavik *et al*. (2013) and Galeuchet *et al*. (2005) studied a more common perennial where there was more evidence of gene flow. Mix *et al*. (2006) found high levels of gene flow in a wind dispersed species despite its recently fragmented distribution.

The present study investigated genetic diversity and population structure in *Rhinanthus minor* which is a representative species of meadow habitats but is an annual so reflects change since fragmentation more readily than a long-lived perennial species (Westbury, 2004). It is also a species which was once much more abundant but is now less common because of habitat loss (Blažek and Lepš, 2015; Linusson *et al*., 1998) Species which have experienced this type of change are under-represented in studies of the impact of fragmentation (Angeloni *et al.*, 2011; Honnay and Jacquemyn, 2007) as are short- lived species (Aguilar *et al*., 2008). This study examined genetic diversity in protected meadow sites which are all managed on an individual basis for conservation. The study area is an upland area in which species-rich hay meadows are located in some of the valleys whilst other fragments of moderately species rich grassland are also distributed throughout the region. These grassland sites have no statutory protection but they may play a role in the exchange of genetic material as part of a network of sites. Small fragments can be important as sources of genetic variation (Gómez-Fernández *et al*., 2016; Van Rossum *et al*., 2004) and this study will assess the extent to which the intermediate grassland fragments contribute to genetic connectivity between the protected meadow sites.

Microsatellite markers have been used in many studies of plant population genetics because of their distribution throughout the genome, high level of polymorphism and co-dominance (Agarwal *et al*., 2008). Microsatellites have been developed for several species of *Rhinanthus* (Ducarme *et al*., 2008; Houston and Wolff, 2009; Talve *et al.*, 2013) and used in a number of studies of population genetics (Hargreaves *et* al., 2015; Houston and Wolff, 2012; Talve *et al*., 2014). This meant that primers were easily available and that there was potential to compare the results from the present study with data from previous studies, and so they were seen as the most appropriate choice for this study.

The study will address the following research questions:

1. What is the level of genetic variation within populations of *Rhinanthus minor* in the meadow sites?
2. Is there any evidence of population differentiation between meadow sites, particularly among those which are more isolated?
3. Do other grassland fragments play a role as a source of genetic material?
4. What are the implications for conservation?

Methods

Study species

Rhinanthus minor L. (Fig. 4.1) is an annual hemi parasitic herb which has a widespread distribution in the UK and much of Europe and North America (Westbury, 2004). It is found in range of grassland habitats but is most commonly associated with meadows (Coulson *et al*., 2001). More intensive grassland management, such as that which requires an early cut for silage, limits the ability of seed production so *R. minor* has seen a decline with the changes in agricultural practices since the middle of the 20th century (Online Atlas of the British Flora, 2017). It is a diploid species (2n = 22) and is either insect- or self-pollinated with the main pollinators being *Bombus* spp. (Natalis and Wesselingh, 2012). Seeds do not persist in the seed bank and seed dispersal is poor with most seeds located <1.5m from the parent plant (Bullock *et al*., 2003; Westbury, 2004).

Fig 4.1 *Rhinanthus minor* in meadow vegetation

Study area and sampling design

The study area is the Forest of Bowland in NW England. This is an upland area with species rich meadows found in the valleys. Information about the climate is given in Chapter 2. Nine of the meadows are protected sites and these were selected as the study sites. Five of the meadows are located within 2 km of each other whilst the other four are more isolated (see Table 4.1). All of the meadows are managed for a field dried hay crop and are mown once a year after mid-July. They are all managed by different farmers.

Table 4.1 Size, elevation and distance to nearest protected meadow for Bowland study sites

Site ID	Size (ha)	Altitude (m a.s.l)	Nearest protected meadow (km)
BG	5.47	180	1.97
BS (3 fields)	7.65	150	0.52
CB	0.54	60	3.49
FH	1.63	105	3.49
FHM	3.33	210	0.46
LCM	5.26	190	0.46
MM (2 fields)	9.09	155	1.29
NI	2.09	125	6.1
TB (5 fields)	11.87	155-180	11.59

m a.s.l = metres above sea level

The areas surrounding the nine main meadow sites were surveyed to locate any other grasslands in which *R. minor* was present. These 'intermediate sites' included other agricultural grasslands, roadside verges, a churchyard and other grassland fragments alongside footpaths and tracks. The intermediate sites were located by using existing knowledge of other species rich grassland sites and by walking the footpaths and roads around the meadows to locate *R. minor* populations. The same survey method was applied throughout the region. Very few populations of *R. minor* were found away from the cluster of sites around Slaidburn (see map, Fig 4.2).

In the main meadow sites leaf samples were collected in 2013 and 2014 from individuals spread evenly across the site at least 5m apart by collecting one sample from each corner of a 5m x 5m quadrat. 20 individuals were sampled in each field by using 5 of these 5m x 5m plots. Some of the

sites have multiple fields in which case each field was sampled. In some sites *R. minor* was infrequent so a smaller number of samples was collected (see Table 4.2). In the intermediate sites transects were used for sampling. Where the intermediate site was close to a main meadow site, the transect was located a minimum of 100m from the main site. Samples were collected at 5m intervals along the transect. This sampling design for the intermediate sites was chosen to accommodate linear features such as roadside verges.

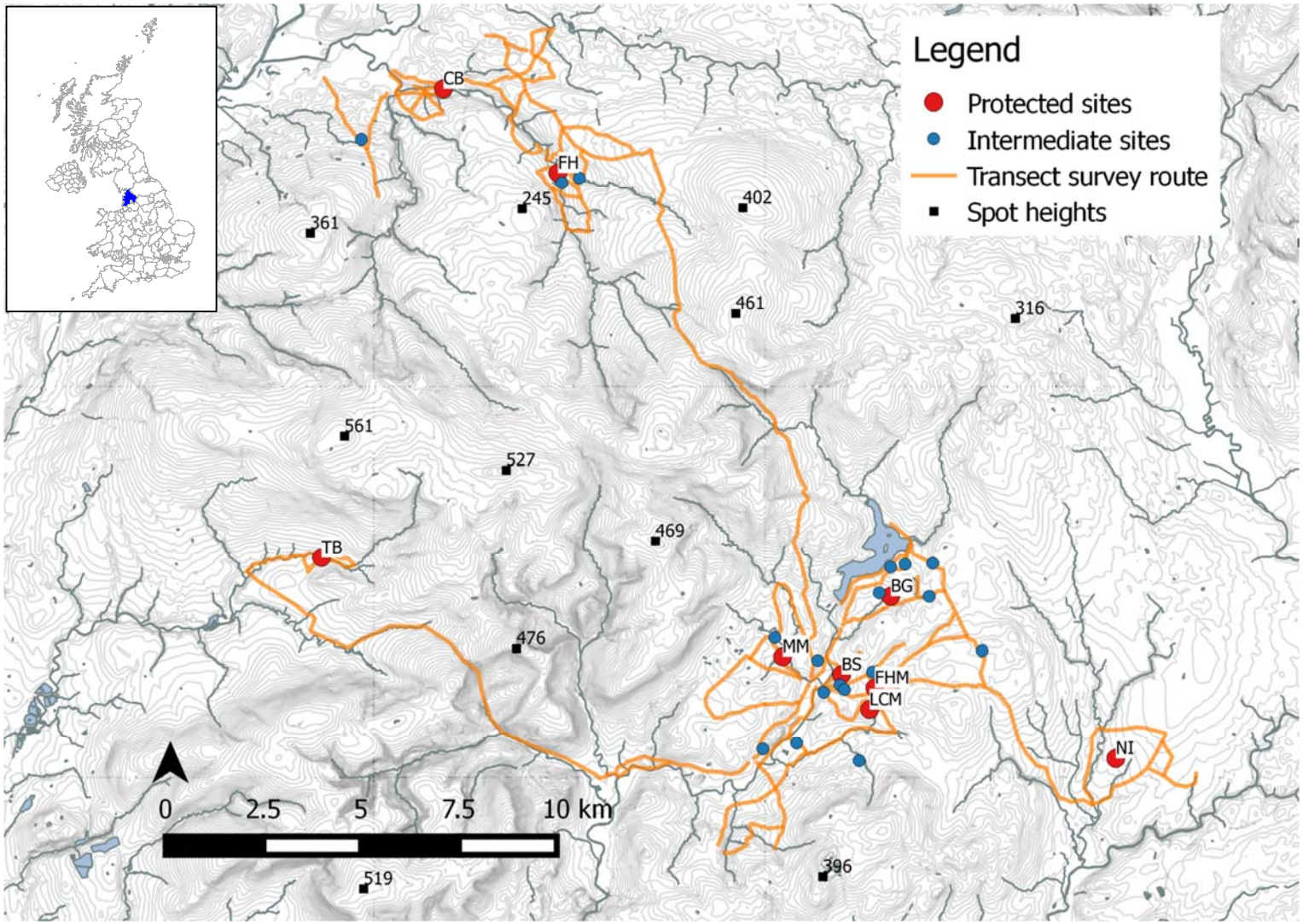


Fig. 4.2 Map showing locations of protected meadow sites, intermediate sites and survey route to identify intermediate sites. Sites < 2km to Slaidburn village are BG, BS, FHM, LCM and MM.

DNA extraction and microsatellite analysis

Leaf samples were stored in silica gel prior to DNA analysis. DNA was extracted following the crude sample PCR protocol supplied with the KAPPA3G plant PCR kit (KAPPA Biosystems, 2016). An approximately 2.5 x 2.5 mm piece was cut from each leaf sample using sterile forceps and added to 50 µl of an extraction buffer containing: 50 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA along with 2% ß-mercaptoethanol and 1.0 mM TCEP before heating at 95°C for 5 minutes.

Six microsatellite primer pairs were used in the analysis. Initial primer testing was carried out on eight primers which were developed for *R. minor* specifically (Houston and Wolff, 2009). Of these, two (RM20 and RM24) were successfully amplified and were found to provide consistent results. Testing was also carried out on six primers which were developed for *Rhinanthus angustifolius* but were also effective in cross species amplification (Ducarme *et al*., 2008). Four of these (RA53, RA75, RA81 and RA87) were used to amplify DNA from *R. minor* successfully in the present study. RA53, RA75 and RA81 were multiplexed using 6-FAM, ATTO 532N and ATTO 565N dyes respectively (Integrated DNA Technologies, Leven, Belgium). RM20, RM24 and RA87 were tagged with 6-FAM (Integrated DNA Technologies) and were amplified in individual reactions.

PCR was carried out in 10 µl reactions containing: 1.0 µl template DNA, 5.0 µl Bioline My Taq Plant Kit (Bioline Reagents Ltd, London, UK), 0.1 µM of each primer pair and 0.2 µl TCEP. Forward primers were fluorescently tagged. The PCR programs followed those in Houston and Wolff (2009) for RM20 and RM24 and Ducarme *et al*. (2008) for RA53, RA75, RA81 and RA87.

The PCR products were diluted 1:4 using nuclease-free H_2O after which 1.0 µl of the diluted PCR product was added to 8.9 µl formamide and 0.1 µl of Applied Biosystems GeneScan 500 LIZ size standard (Applied Biosystems, Warrington, UK). Fragment analysis was undertaken using capillary

electrophoresis on an AB 3500 Genetic Analyzer (Applied Biosystems, Warrington, UK) and scored using Gene Mapper 5.0 software (Applied Biosystems, Warrington, UK) with manual checks carried out for scoring errors. Allele binning was carried out using the TANDEM program (Matschiner and Salzburger, 2009).

Data analysis

Data analysis was carried out in R version 3.3.2 (R Core Team, 2016).

To estimate genetic diversity calculations of observed heterozygosity (H_o), expected heterozygosity (H_e) and allelic richness (A) were calculated by site (population) and for the dataset as a whole (region) using the poppr package version 2.3.0 (Kamvar *et al*., 2014). Allelic richness was corrected for sample size by rarefaction using the R package hierfstat (Goudet and Jombart, 2015). The relationship between site size in hectares and genetic diversity (using expected heterozygosity as a measure) was tested using a Spearman Rank correlation test. A parametric test was not used because the data were not normally distributed. Deviation from Hardy-Weinberg Equilibrium (HWE) was tested using $\chi 2$ over all loci with a Monte Carlo permutation test (999 iterations) for significance. To assess whether populations were in linkage disequilibrium the $\overline{r}_d$ index (Agapow and Burt, 2001) was used in the poppr package. This is an index of association which accounts for the number of loci sampled and can be tested for significance using a permutation test (999 iterations were used). Private alleles were identified in the meadows and intermediate sites using the poppr package.

The inbreeding coefficient (F_{IS}) was calculated for both clone-corrected and uncorrected data because of the potential for *R. minor* to be self-pollinated. Differences between clone-corrected and uncorrected values were investigated using a paired *t*-test The poppr package allows for clone correction on the basis that one individual per multilocus genotype is represented in each population (Kamvar *et al*., 2014). F_{IS} values were calculated in the adegenet package and were based on mean values from the likelihood distribution of each individual (Jombart, 2017).

Population differentiation was analysed using pairwise F_{ST} values for each population, along with a global estimate of overall genetic differentiation in the package hierfstat. The significance of the overall F_{ST} value was tested using a Monte Carlo procedure with 99 permutations. It has been suggested that that F_{ST} may over- or under-estimate population differentiation (Gregorius, 2010; Jost, 2008). Alternative measures have been proposed so Hedrick's standardised $G"_{ST}$ was also calculated because it accounts for demographic processes such as genetic drift and migration on population structure (Meirmans and Hedrick, 2011). Calculations were made in the mmod package (Winter, 2012; Winter, in press).

To investigate the relationship between genetic dissimilarity and geographical distance between populations (isolation by distance) distance-based redundancy analysis (dbRDA) was used (Legendre and Anderson, 1999). This method has been used successfully in the analysis of genetic data (Vangestel *et al*., 2012) and has been proposed as an alternative to the Mantel test for several reasons including flexibility in the use of different types of explanatory variables and greater statistical power (Kierepka and Latch, 2015; Legendre and Fortín, 2010). dbRDA was performed in the vegan package in R (Oksanen *et al*., 2016) using the capscale function. dbRDA is an ordination method in which a principal co-ordinates analysis is performed on a dissimilarity matrix of the response data (the percentage dissimilarity of shared alleles in this study) and a redundancy analysis is then carried out. The explanatory variable in the RDA was the spatial data or geographical co-ordinates of each sample. The vegan package includes a permutation test to analyse the significance of the relationship. dbRDA was performed on the data from the meadow sites and, in a separate analysis, on the intermediate sites. The analysis is carried out on an individual rather than population basis so the small sample sizes in the intermediate sites would not affect the result.

The spatial genetic structure of the populations was explored by performing a spatial principal components analysis (sPCA) (Jombart *et al*., 2008) using the R package adegenet (Jombart, 2008). PCA can be used to summarise genetic variability among individuals but sPCA takes this further by incorporating spatial patterns of genetic variability (Jombart *et al*., 2008) The principal component scores of allele frequencies for each individual are multiplied with Moran's *I* which is a measure of spatial autocorrelation. Statistical tests, using a Monte Carlo permutation procedure can be performed to test for global and local structures. Where there is global structure there is a high degree of spatial autocorrelation and individuals are likely to be genetically similar to their neighbours. The presence of local structure indicates negative autocorrelation and genetic dissimilarity (Warren *et al*., 2016). The adegent package in R enables the use of different connection networks to be used in the analysis. A Delaunay triangulation network was used because it covers more connections between sites and empirical data suitable for a distance-based neighbour network were not available. The sPCA eigenvalues were examined to determine how many axes should be retained.

A sPCA analysis was also performed on the data from the intermediate sites. sPCA can be used to identify patterns in individual as well as population data and the intermediate site data were analysed on an individual basis. The analysis investigated whether populations of *R. minor* in small grassland fragments and non-protected sites had similar genetic patterns to the protected sites or displayed some genetic differentiation. Finally, a sPCA was performed on the meadow and intermediate sites together to establish if the same patterns of differentiation were evident when an overall analysis was undertaken.

The results of the sPCA using the lagged scores were displayed using the colorplot function in the adegenet package. The lagged scores were used because they reduce the 'noise' in the data are better for identifying global structures (Jombart, 2017), and the colorplot function translates each score into

a colour such that the different shades of the red, green and blue colour system give an indication of genetic differentiation with similar colours representing genetic similarity.

Results

Genetic diversity

Genetic diversity parameters are shown in Table 4.2. Observed heterozygosity was lower than expected heterozygosity in all populations and none of the loci were in HWE (Table 4.3). The correlation between site size and expected heterozygosity was positive and significant at the ≤ 0.05 significance level (rho = 0.68, S = 38, p-value = 0.050). Tests for linkage equilibrium did not reveal significant results for any of the populations (clone corrected data). Expected heterozygosity by population ranged from 0.034 to 0.507 but the overall value was 0.472. Levels of inbreeding were relatively high with the overall F_{IS} value slightly higher in the clone-corrected data. There were differences between population data for uncorrected and clone-corrected values which were statistically significant at the the ≤ 0.05 significance level when a paired *t*-test was undertaken (t = 2.3035, df = 8, P = 0.050). Private alleles were identified in two of the meadow populations. In population BS four homozygotes and one heterozygote were not found anywhere else in the meadows or intermediate sites. All of these were in one of the four fields sampled at this site. There was one unique allele in population G and one in intermediate site HL. Two individuals had private alleles in intermediate site HHL, one a heterozygote and the other a homozygote.

Table 4.2 Genetic diversity parameters by population

Site code	*N*	*A*	H_o	H_e	F_{IS}	F_{IS} (cc)
BG	21	2.9	0.151	0.450	0.582	0.570
BS	88	3.13	0.127	0.507	0.620	0.619
CB	12	2.27	0.247	0.408	0.460	0.447
FH	20	1.24	0	0.034	0.665	0.664
FHM	21	2.21	0.26	0.359	0.457	0.465
LCM	18	2.51	0.089	0.339	0.604	0.595
MM	40	2.71	0.136	0.441	0.591	0.569
NI	12	2.26	0.059	0.390	0.677	0.643
TB	22	2.80	0.179	0.463	0.565	0.562
Overall	254	2.45	0.139	0.472	0.648	0.656

N = sample size, *A* = rarefied allelic richness, H_o = observed heterozygosity, H_e = expected heterozygosity, F_{IS} = inbreeding coefficient, F_{IS} (cc) = clone corrected inbreeding coefficient.

Table 4.3 Result of Chi-squared test for deviation from Hardy-Weinberg equilibrium for all populations by loci

Locus	χ^2	df	Significance
RM20	524.72	15	***
RM24	690.84	10	***
RA53	1147	28	***
RA75	166.32	1	***
RA81	485.14	6	***
RA87	100.98	3	***

*** represents *P* values of <0.001 following a Monte Carlo test with 999 permutations.

Population differentiation

The overall F_{ST} value was 0.141. This value was found to be significantly different from zero in a Monte Carlo permutation test ($P = 0.01$). Pairwise F_{ST} values are given in Table 4.4. Pairwise values ranged from 0.011 to 0.420. Population FH was found to have substantially higher values than the other populations. FH had a substantial number of clones among the sampled individuals so a clone corrected F_{ST} was calculated and this reduced the overall value to 0.072 although it was still significantly different to zero ($P = 0.01$). Hedrick's standardised G''_{ST} was calculated for the uncorrected data $G''_{ST} = 0.319$ and for the clone-corrected data $G''_{ST} = 0.252$.

Table 4.4 Pairwise F_{ST} values (clone corrected) by population

	BS	CB	FH	BG	FHM	LCM	MM	NI
CB	0.017							
FH	0.045	0.018						
BG	0.114	0.041	0.238					
FHM	0.055	0.041	0.075	0.177				
LCM	0.096	0.029	0.132	0.187	0.135			
MM	0.024	0.030	0.045	0.092	0.028	0.053		
NI	0.037	0.009	0.023	0.238	0.075	0.107	0.037	
TB	0.072	0.023	0.032	0.169	0.109	0.111	0.082	0.038

Spatial analysis

The dbRDA revealed that there was a significant pattern of isolation by distance in the Bowland meadow populations ($R^2_{adj} = 0.001$, $F = 1.202$, df. = 2, $P = 0.001$). However the low adjusted R^2 value shows that this a weak relationship. The ordination plot (Fig. 4.3) shows little separation between populations although one site (TB) is distinct from the others. This site is the most geographically isolated. For the intermediate sites there was again a significant but weak relationship

(R^2_{adj} = 0.001, F = 1.604, df. = 2, P = 0.001). In the ordination plot only two colours were assigned to identify samples from the sites close to the Slaidburn meadows or those in the NW of the region. Assigning colours to all the grassland fragments would have made the plot difficult to interpret. Fig. 4.4 shows some evidence of isolation by distance between the Slaidburn sites and the more isolated NW grasslands.

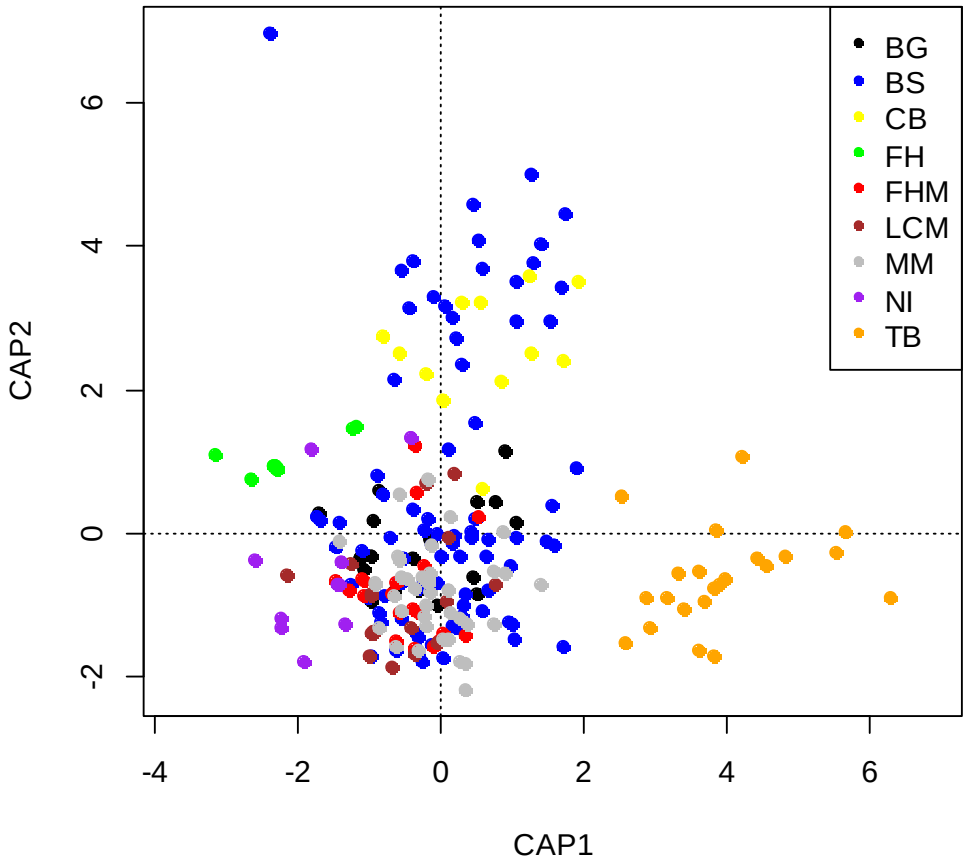


Fig. 4.3 Ordination plot of distance-based Redundancy Analysis of genetic dissimilarity constrained by geographic distance in the Bowland meadows. Each meadow is represented by a different colour.

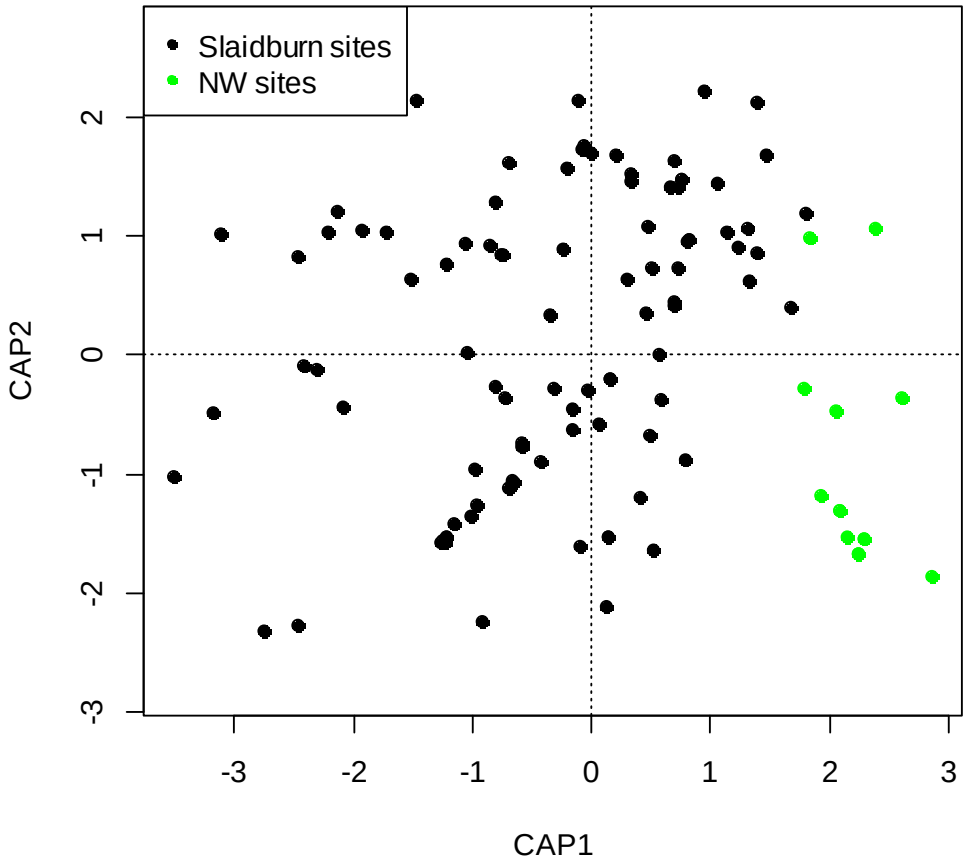


Fig. 4.4 Ordination plot of distance-based Redundancy Analysis of genetic dissimilarity constrained by geographic distance in the intermediate sites. Samples from sites close to the Slaidburn meadows (see map Fig 4.2) are represented by black dots and sites in the NW of the region by green dots.

The test for global and local structures in the sPCA revealed a significant global structure [max(t) = 0.022, P = 0.002] but did not show evidence of local structure [max(t) = 0.013, P = 0.601]. However the colorplot representation did not indicate any clear pattern of differentiation with the exception of one site - BG (Fig. 4.5) The sPCA for the intermediate sites again revealed a significant global structure [max(t) = 0.062, P = 0.001] and did not show evidence of local structure [max(t) = 0.014, P = 1]. The colorplot (Fig. 4.6) showed that there was spatial differentiation in the intermediate sites and confirmed the isolation by distance shown in the dbRDA. When all of the meadows and

intermediates sites were analysed in a sPCA the result indicated both global [max(t) = 0.013, P = 0.001] and local structure [max(t) = 0.009, P = 0.005]. The colorplot (Fig. 4.7) suggested that there was gene flow between the Bowland sites but confirmed the local differentiation of site BG and the intermediate sites close by, and showed some differentiation in intermediate sites close to FH.

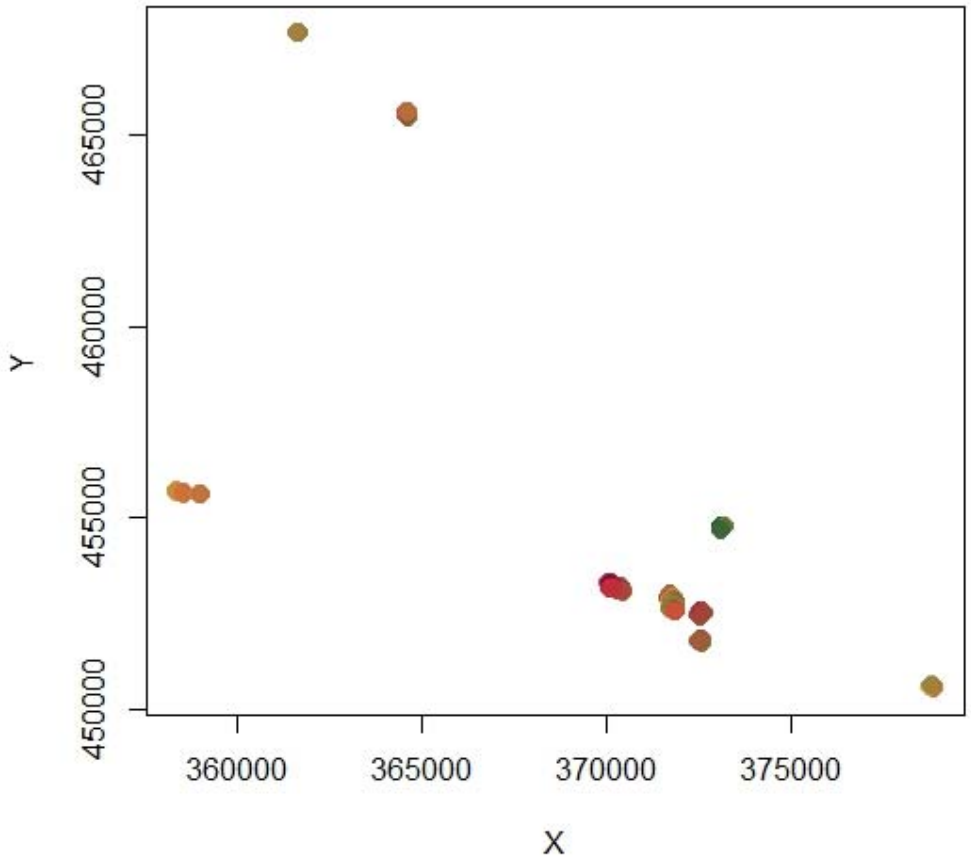


Fig. 4.5 Colorplot of Bowland meadow sites sPCA using lagged scores with the first two eigenvalues retained.

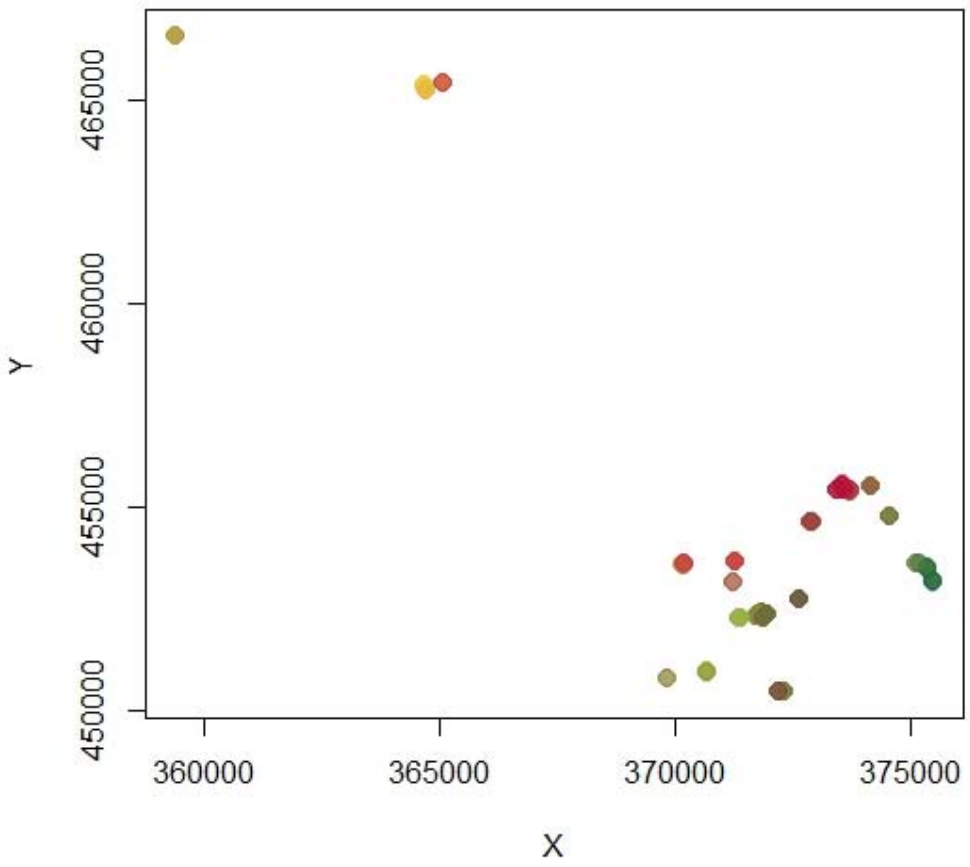


Fig. 4.6 colorplot of intermediate sites sPCA using lagged scores with the first two eigenvalues retained and Delaunay connection network.

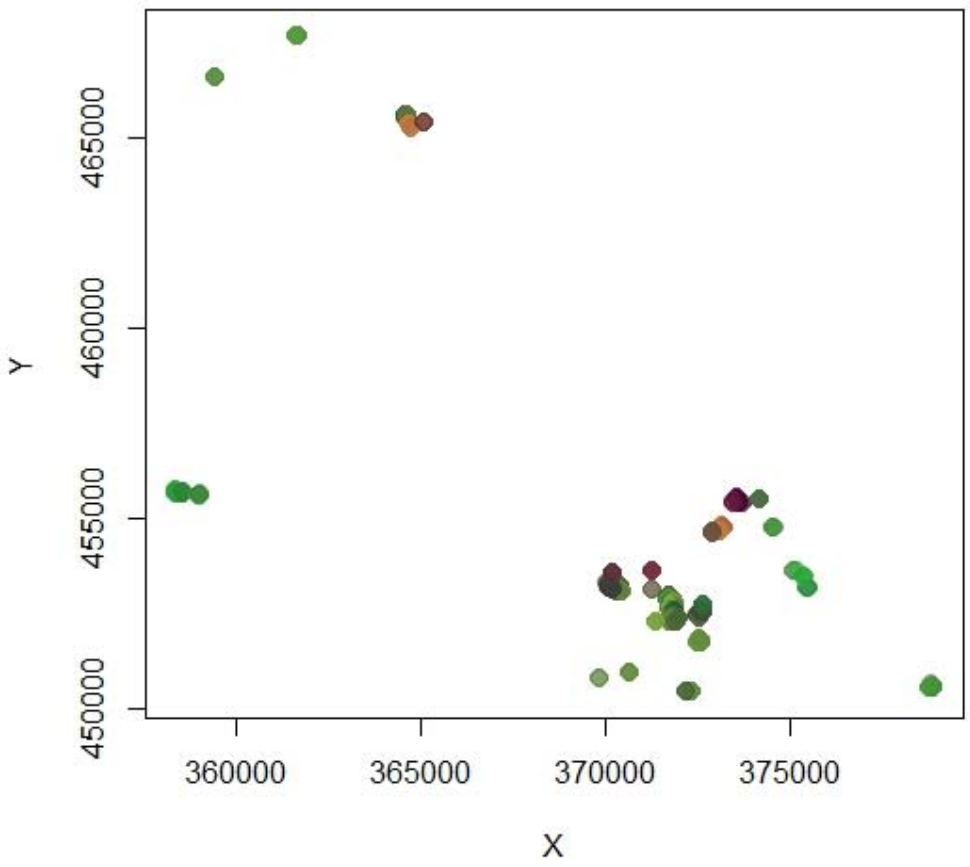


Fig. 4.7 colorplot of meadow and intermediate sites sPCA using lagged scores with the first two eigenvalues retained and Delaunay connection network.

Discussion

This analysis of *R. minor* in the Bowland meadows has revealed moderate levels of genetic diversity with no major differences between diversity in individual sites but with high levels of inbreeding. There was evidence of gene flow between the meadows but some patterns of local scale differentiation exist in sites that were not particularly isolated. The intermediate sites, including small populations in areas such as roadside verges, played a role in genetic connectivity in the area.

Genetic diversity and inbreeding

Moderate levels of genetic diversity were found in all the *R. minor* meadow populations except population FH where the high proportion of clones indicates high rates of selfing. The analysis showed that there was a positive relationship between the size of the site in area and expected heterozygosity, and FH was one of the smallest sites. Leimu *et al*. (2006) found that there was a positive relationship between population size and expected heterozygosity but population size does not necessarily equate to site size. Figure 4.4 indicates that the relationship between site size and population differentiation is not very evident in the spatial analysis. Expected heterozygosity in the Bowland populations was similar to a study of *R. minor* populations at locations across the UK in which H_e ranged from 0.35 to 0.53 (Houston and Wolff, 2012), compared with Bowland H_e values of 0.034 to 0.507. Values in the Bowland populations were lower than those found in studies of predominantly outcrossing perennial plants (Aavik *et al*., 2013; Suni and Whitely, 2015) and in study of a polyploid species (Van der Meer and Jacquemyn, 2015) which was in line with expectations. Low values of observed heterozygosity meant that none of the populations were in Hardy-Weinberg equilibrium.

Low values of observed heterozygosity could be explained by the high levels of inbreeding in the Bowland populations, a finding which was also reported in other studies of *R. minor* (Ducarme and Wesselingh, 2013; Talve *et al.*, 2013). However, F_{IS} values were higher in Bowland than in those found by Houston and Wolff (2012) in their UK-wide study (F_{IS} 0 – 0.44 compared with Bowland (F_{IS} 0.44 – 0.66). Inbreeding is predicted to lead to a reduction in fitness and to be more problematic in small populations where individuals are more likely to mate with others with a recent common ancestry (Lienert, 2004; Young *et al*., 1996). However, empirical evidence has shown that the effects of inbreeding are complex. A meta-analysis revealed that inbreeding depression increases with population size but that there was no significant difference in the magnitude of inbreeding depression between self-compatible and self- incompatible species (Angeloni *et al*., 2011). A study of another species in the *Rhinanthus* genus*, R. alectorolophus*, did not find any difference in inbreeding

depression in samples of selfed and open-pollinated plants (Sandner and Matthies, 2017). This finding also coincides with the results of a study by Hargreaves *et al*. (2015) who found little evidence of inbreeding depression in *R. minor* populations despite high rates of selfing. Angeloni *et al*. (2011) warned that inbreeding is widespread in plant populations and that a greater understanding of its effects on plant fitness is required so that conservation can be better targeted.

It is difficult to determine reasons for the high rates of selfing in population FH, which was also found in the intermediate sites close to FH. Pollinator absence could be a possibility but selfing has been shown to occur in *R. minor* when pollinators were recorded as present (Hargreaves *et al*., 2015). Some species of *Bombus* have been shown to trigger self -pollination according to how they land on, and enter the *R. minor* flower (Westbury, 2004). Records from the long-term change study described in Chapters 3 and 4 show that the abundance of *R. minor* at site FH increased from rare to abundant between the first and second surveys which suggests that high levels of inbreeding are not a result of a reduction in population size in this particular population.

Population structure

The analysis of population differentiation based on the relatively low clone-corrected F_{ST} value suggested that there was evidence of gene flow among the populations of *R. minor* in the Bowland region. The $G"_{ST}$ value was higher than the F_{ST} value and this might indicate that there is an underestimation of population structure because the number of populations included in the study is relatively low (Meirmans and Hedrick, 2011). However, the sPCA global structure tests and colorplots (Figure 4.4 and 4.6) indicated that there was not a high degree of population differentiation for the majority of the meadow sites, and that there was gene flow among the *R. minor* populations.

The spatial analysis revealed a pattern of isolation by distance which was particularly pronounced in the intermediate sites, and that one of the Slaidburn sites (BG) and intermediate sites close to BG were differentiated from the other sites. The intermediate sites vary in terms of population size and proximity to other populations of *R. minor* but some are very small population fragments. In the main meadow sites the population sizes are sufficiently large (estimated at 1000 to >10,000 in the larger sites) for it to be unlikely that genetic drift would be a contributor to population differentiation (Willi and Määttänen, 2011; Wright, 1931). However, whilst the small sample sizes make it difficult to draw conclusions in the smaller intermediate sites unique alleles were found at two sites, and it would be expected that such small sites would be more vulnerable to genetic drift (Lienert, 2004).

The analysis identified that there was some local structure, and site BG and the intermediate sites close to it were showing differentiation from the other sites in the Slaidburn cluster and from some of the sites in the NW of the study area, namely the intermediate sites close to FH. FH and BG are approximately 13 km apart and separated by an area of moorland which is > 400 m above sea level so this differentiation could be explained by distance and landscape factors. However, site BG and the surrounding intermediate sites are also showing differentiation from the other Slaidburn meadows and intermediate sites which are approximately 2 km away with an area of more intensively farmed grassland in between. Records from the long-term surveys show that one of the intermediate sites close to BG was only colonised by *R. minor* after 1997 whilst a neighbouring intermediate site had shown a marked increase in *R. minor* abundance. It may be possible that BG has had a different management history or there are other environmental factors such as local differences in aspect, altitude or soil type which are causing local selection pressures, and that the intermediate sites close by have been colonised by offspring from the BG population.

The spatial analysis indicates that there is gene flow in the region in addition to these local patterns of differentiation. The limited seed dispersal exhibited by *R. minor* would suggest that most genetic

connectivity is linked to pollen transport unless there is an anthropogenic influence on seed dispersal. Movements of farm machinery or even shared used of equipment have been shown to facilitate seed dispersal (Bullock *et al*., 2003; Le Corre *et al*., 2014). It is also possible that current genetic patterns are still reflecting the historic pattern of land use to some extent. It has been shown that time since fragmentation, and more importantly for annual species, number of generations since fragmentation occurred have a significant negative effect on genetic diversity (Aguilar *et al*., 2008). It is well known that the decline in species rich grasslands in the UK had begun by the middle of the 20th Century but the timing of site losses would have varied from region to region (Hodgson *et al*., 2005). The long-term survey records include six sites where *R. minor* was present in the 1980s but was absent in the 2012-2014 surveys so some of the losses of populations in this region may have been relatively recent.

Contribution of the intermediate sites to genetic diversity

The sPCA showed that most of the intermediate sites had a similar genetic pattern to the protected meadow sites (apart from site FH). This indicates that there is gene flow between them and that they are contributing to the overall genetic connectivity in the region. However, the significant pattern of isolation by distance suggests that these sites, and particularly the smaller fragments, may be more at risk from the effects of fragmentation.

Implications for conservation

Current levels of genetic diversity would indicate that the impact of fragmentation is not having a particularly detrimental effect on the populations of *R. minor*. However, high levels of inbreeding are a feature of the genetic composition of these populations. The need for a greater understanding of the impact of inbreeding on fitness has already been discussed and this should prompt a precautionary approach to conservation especially where small and isolated populations are concerned. It has been demonstrated that there is gene flow in the Bowland populations so site

fragmentation has not resulted in high levels of population differentiation but there are indications that some sites are differentiated and that isolation by distance is occurring and conservation action should consider how further differentiation could be prevented. The results of this study provide some justification for the adoption of a landscape scale approach which maximises connectivity between sites, including the incorporation of small population fragments which have been shown to contribute to overall genetic diversity.

Chapter 5. A comparison of genetic diversity and population structure in meadow populations of *Rhinanthus minor* in two contrasting regions of the UK.

Introduction

Losses of semi-natural grasslands including species-rich meadows have been particularly severe in lowland areas of the UK and Europe mainly due to the greater suitability for change to intensively managed grasslands and arable crops (Critchley *et al.*, 2004; Hodgson *et al*., 2005; Hooftman and Bullock, 2010). The landscape matrix in which surviving species-rich grassland sites are located can be an important influence on plant species diversity (Schmucki *et al*., 2012) with one study highlighting that the species richness of grasslands was significantly lower in areas dominated by arable land (Öckinger *et al*., 2012). Gene flow among fragmented grassland plant populations in intensively managed agricultural landscapes has been found to be limited in some cases (Vanden Broeck *et al*., 2015; Van Rossum *et al*., 2004) but has been relatively uninhibited in others although this can be linked to traits such as wind-dispersal mechanisms in seeds (Aavik *et al*., 2014; Mix *et al*., 2006).

Studies which increase our knowledge of the impact of agricultural land use on gene flow between fragmented populations are complemented by research which investigates other landscape variables. Variations in topography and altitude and their potential to impact gene flow have also been addressed. In a large-scale study the genetic diversity of populations of plants in mountainous regions was not found to be related to elevation but the effects of topography and the physiography of mountain ranges were important influences (Thiel-Egenter *et al*., 2009). Plant populations in Alpine regions can be naturally isolated but even in upland areas with much lower elevations genetic

diversity and gene flow were greater at higher altitudes for the insect pollinated plant *Geranium sylvaticum* (Ernst *et al*., 2013).

Studies which compare genetic variation in similar habitats but contrasting landscape contexts are rare. The conservation approach for hay meadows varies little across the UK with the majority of species-rich sites being managed through agri-environment schemes (Mountford *et al*., 2013). However, evidence that fragmented sites have equal levels of genetic connectivity in different regions is lacking when it may be that some regions are more at risk from fragmentation than others and this may have important implications for conservation.

One of the conservation responses to the fragmentation of species-rich grassland has been to restore grasslands with a lower botanical diversity by introducing seed from other sources (Westbury *et al*., 2006). There has been debate about the importance of the provenance of seed sources for restoration with contrasting views on the need for locally sourced seed (Bischoff *et al*., 2010; Reiker *et al*., 2015). Houston and Wolff's (2012) study of *R. minor* populations across the UK concluded that local seed sources were not necessary because there was no evidence of isolation by distance within UK populations. The investigation of the Bowland meadows revealed some isolation by distance but focused on sites from only one region. A comparison with another region of the UK will provide information about population differentiation and isolation by distance at a larger scale and will give the genetic patterns some context.

The analysis of genetic variation in the Bowland meadows presented in Chapter 4 revealed that there was gene flow in the region but that there was also some local-scale differentiation. This chapter will consider whether such patterns of genetic variation are repeated in other populations by investigating genetic variation in *Rhinanthus minor* in meadows in Worcestershire, a lowland region which is approximately 200 km to the south of the Bowland sites. The Worcestershire area was selected because there is a relatively high number of species-rich meadows from which study sites could be

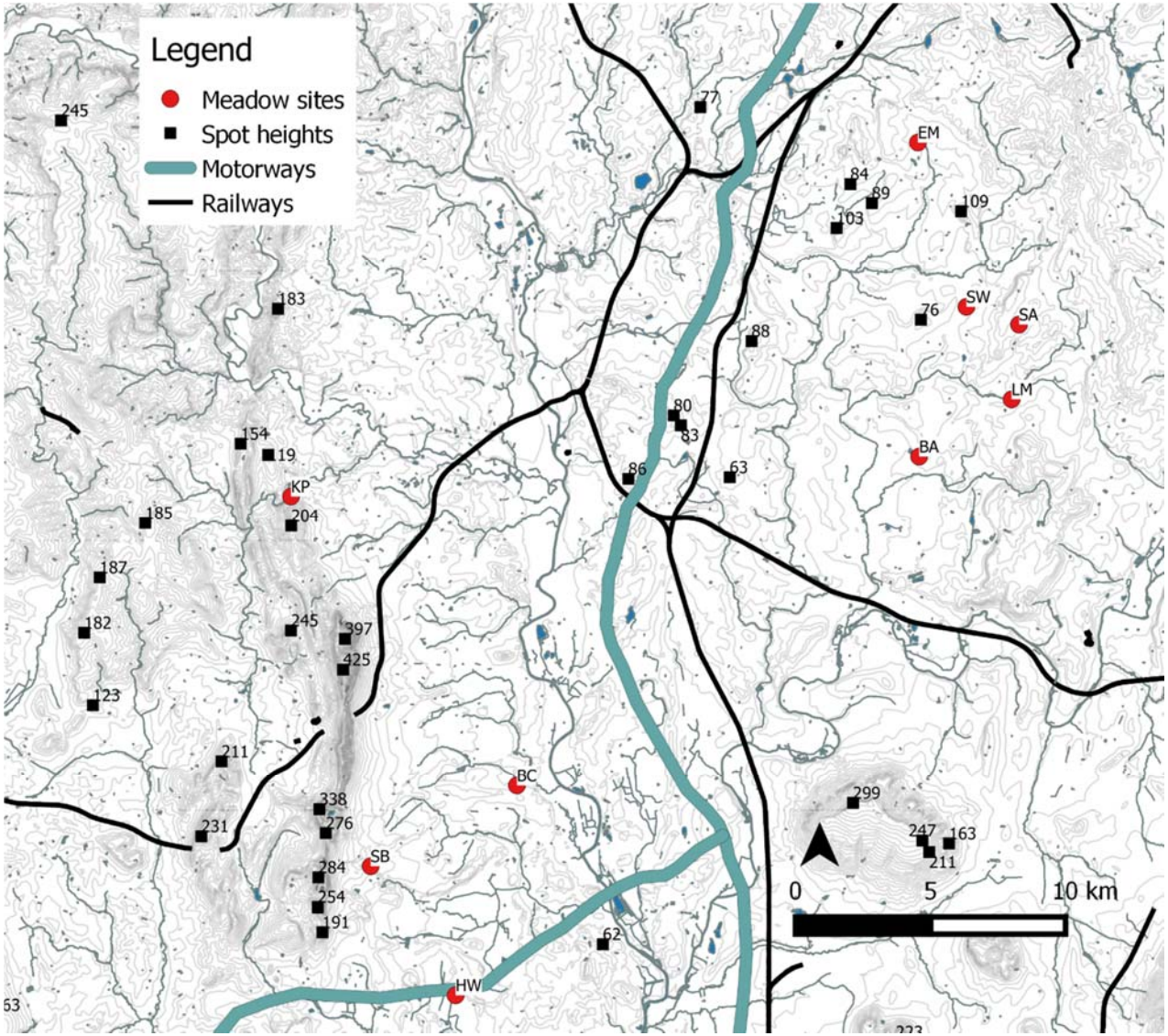
chosen, and because it has contrasting topography and land use to Bowland. A comparison of genetic diversity within the two regions will be made, and the extent of population differentiation between the regions will be examined. This chapter will address the following research questions:

1. How genetically diverse are the *Rhinanthus minor* populations of lowland meadows in Worcestershire and do they differ from that of upland meadows in Bowland?
2. Are the meadow populations of *R. minor* genetically differentiated in Worcestershire and how does this compare to the findings in the Bowland meadows?
3. Is there a significant pattern of population differentiation between the two regions?
4. What are the implications for conservation?

Methods

Study area

Meadow sites were sampled in the Forest of Bowland in NW England and in Worcestershire in Central England. The map in Figure 5.1 shows the locations of the Worcestershire sites. A map of the Bowland sites is in Figure 4.2, Chapter 4. The two regions are located approximately 200km apart and have contrasting land-use and topographical characteristics. Approximately 25 % of the farmed area is occupied by cereal crops and 45 % by permanent grassland in the areas of Worcestershire in which the sample sites are located (Worcester and Wychavon; Malvern Hills Districts). In the Bowland sampling areas (Ribble Valley and Lancaster Districts) cereals occupy < 1% of the farmed area and permanent grassland makes up > 95% (Source: DEFRA, 2017). The density of protected meadow sites in Bowland (9 sites) is lower than in the Worcestershire sampling areas (approximately 30 sites) despite the fact that the landscape is less intensively farmed.



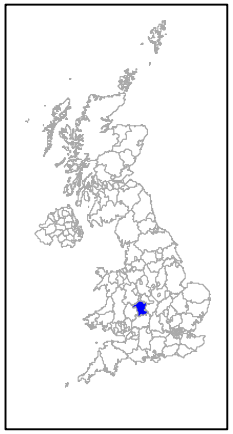


Fig. 5.1 Map showing location of Worcestershire meadow sites. Sites BA, EM, LM, SA and SW are in NE of sampling area. Sites BC, HW, KP and SB are in SW of sampling area. Motorways and railways are shown.

Nine meadows from each area were included all of which are managed as agricultural grasslands but are either protected sites and/or are under an agri-environment scheme management agreement. Details of the sites are shown in Table 5.1.

Table 5.1 Details of the Bowland and Worcestershire sites.

Bowland sites	Altitude (m. a.s.l)	Size (ha)	*N*	Nearest protected meadow	Nearest *R. minor* pop	*A*
BG	180	5.47	21	1.97	0.1	2.91
BS	150	7.65	88	0.52	0.1	3.13
CB	60	0.54	12	3.49	2.45	2.27
FH	105	1.63	20	3.49	0.1	1.24
FHM	210	3.33	21	0.46	0.46	2.21
LCM	190	5.26	18	0.46	0.46	2.51
MM	155	9.09	40	1.29	0.2	2.70
NI	125	2.09	12	6.1	4.3	2.26
TB	155-180	11.87	22	11.59	11.0	2.80
Worcestershire sites	Altitude (m. a. s. l.)	Size (ha)	*N*	Nearest protected meadow	Nearest study site	*A*
BA	35	1.07	27	1.6	3.8	2.42
BC	20	6.84	32	1.3	6.1	2.89
EM	85	7.0	32	2.2	6.1	2.66
HW	15	11.41	32	0.8	5.5	2.74
KP	75	3.91	32	2.4	13.5	2.88
LM	50	5.12	32	2.6	2.7	1.89
SA	90	4.67	32	3.1	2.0	2.43
SB	45	2.21	30	1.9	5.5	2.39
SW	55	1.75	32	1.50	2.0	2.62

m. a. s. l = metres above sea level; ha = hectares; *N* = number of samples; Nearest protected meadow = closest protected meadow site in km. Nearest *R. minor* pop = closest intermediate site in Bowland survey in km; *A* = rarefied allelic richness; Nearest study site = distance in km to closest meadow site included in Worcestershire study.

The sampling method was adapted slightly from that used in the Bowland meadows to try to achieve more even sample sizes in the Worcestershire study. When there were multiple fields in the Bowland

sites each field was sampled separately and 20 samples were collected in each field. However, since there was found to be little variation across these multiple fields, and the original method resulted in small sample sizes in some of the sites where *R. minor* was not as frequent, the method was changed. Plots measuring 5 m by 5 m were marked out in 8 areas covering all areas of each site regardless of whether there were multiple fields where *R. minor* was present. Leaf samples were taken in May 2017 from an individual at each corner of the plot and stored in silica gel as they were for the Bowland samples. This resulted in sample sizes of 32 for all sites except for sites BA and SB where *R. minor* was less abundant. Laboratory methods followed those described in the Chapter 4.

Data analysis

To compare genetic variation between the Bowland and Worcestershire *R. minor* data calculations of observed heterozygosity (H_o), expected heterozygosity (H_e) and rarefied allelic richness (A) were calculated by site (population) and for the dataset as a whole (region) using the poppr package version 2.3.0 (Kamvar *et al*., 2014). The inbreeding coefficient (F_{IS}) was calculated for both clone-corrected and uncorrected data using the same methods as that for the Bowland data. Tests for linkage disequilibrium and the identification private alleles were carried out using the methods described in Chapter 4. A Spearman Rank correlation was used to test for a relationship between size of site in hectares and genetic variation (measured by expected heterozygosity, H_e. The measures of genetic diversity and inbreeding for the Bowland and Worcestershire meadows were compared using Wilcoxon rank sum or two sample *t*-tests depending on the outcome of checks for normality of distribution and heterogeneity of variance.

Estimates of population differentiation were calculated using F_{ST} and pairwise F_{ST} and Hedrick's standardised G''_{ST} as they were for the Bowland meadows with comparisons being made between the results for each region. The significance of the overall F_{ST} value was tested using a Monte Carlo procedure with 99 permutations as it was for the Bowland data. Overall F_{ST} and Hedrick's

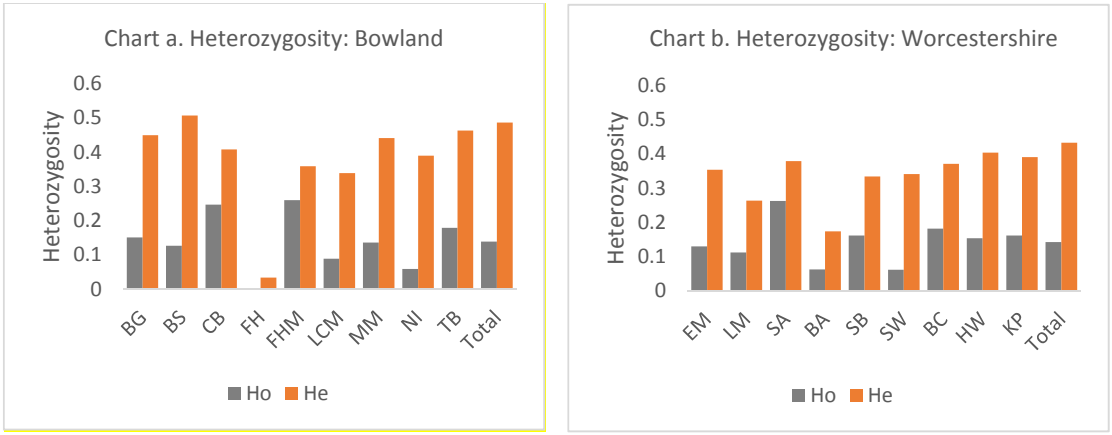
standardised G''_{ST} values were calculated for the combined Bowland Worcestershire data to assess differentiation between the two regions.

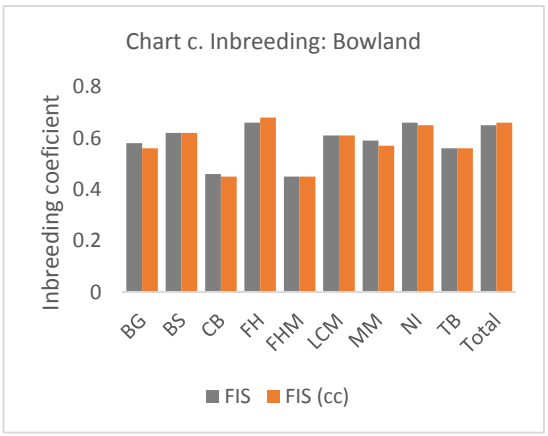
For the spatial analysis of the Worcestershire data a similar approach was taken to that used in Chapter 4. Isolation by distance was tested using dbRDA then the same analysis was undertaken with a combination of the Bowland and Worcestershire data. A sPCA of the Worcestershire data was carried out along with an analysis of the combined Worcestershire and Bowland data. Permutation tests were used to assess the significance of the dbRDA and for global and local structure within the sPCA. Methods for the dbRDA and sPCA were described in detail in Chapter 4.

Results

Genetic diversity and inbreeding

Estimates of genetic diversity in the Bowland and Worcestershire meadows are shown in Figure 5.2 a-d. Expected heterozygosity (H_e) in the Worcestershire meadows ranged from 0.174-0.404 and observed heterozygosity (H_o) ranged from 0.063 to 0.263. None of the loci were in Hardy-Weinberg equilibrium when a chi squared test was carried out (Table 5.2). Levels of inbreeding were relatively high ranging from 0.44 (0.45 clone-corrected) to 0.64 (0.63 clone-corrected). The correlation between site size and H_e was positive but not significant for the Worcestershire data (rho = 0.58, S = 50, P = 0.108) where the result for the Bowland meadows showed a slightly stronger correlation which was close to the significance level of < 0.05 (rho = 0.68, S = 38, p-value = 0.050). None of the Worcestershire measures of diversity were significantly different from the Bowland measures when t – Tests and Wilcoxon rank sum tests were performed (Table 5.3).





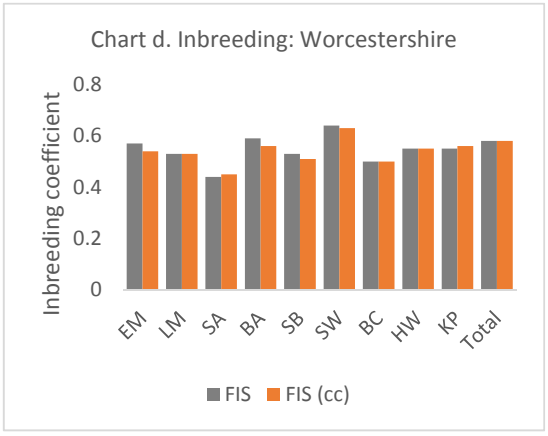


Fig. 5.2 Bar charts showing heterozygosity (Charts a and b) and inbreeding (Charts c and d) in the Bowland and Worcestershire meadows. Charts show values by site (population) and an overall figure (represented by the 'total' column on the chart)

Table 5.2 Results of a chi-squared test for Hardy-Weinberg equilibrium over all loci of the Worcestershire data

Locus	χ^2	df	Significance
RM20	467.57	10	***
RM24	522.03	15	***
RA53	1329.11	36	***
RA75	209.50	10	***
RA81	186.11	3	***
RA87	340.37	3	***

Monte Carlo permutation procedure (999 permutations) used to test for significance. *** represents *P* values of <0.001.

Table 5.3 Results of tests for difference between genetic diversity parameters for the Bowland and Worcestershire (Worcs.) data

	Test statistic	Mean (SE) Bowland	Mean (SE) Worcs.	*P* value
H_e	W = 60			*P* = 0.094 (NS)
H_o	*t* = -0.13, df = 15	0.14(0.03)	0.14(0.02)	*P* = 0.895 (NS)
A	*t* = -0.46, df = 13	2.45(0.18)	2.55(0.1)	*P* = 0.652 (NS)
F_{IS}	*t* = 1.02, df = 15	0.58(0.03)	0.54(0.02)	*P* = 0.326 (NS)
F_{IS} (cc)	*t* = 1.13, df = 13	0.57(0.03)	0.54(0.02)	*P* = 0.278 (NS)

W = test statistic for Wilcoxon rank sum test; *t* = test statistic for two sample *t*-Test; H_e = expected heterozygosity; H_o = observed heterozygosity; A = rarefied allelic richness; F_{IS} = inbreeding coefficient; F_{IS} (cc) = inbreeding coefficient for clone corrected data; SE = standard error. NS = not significant.

Population differentiation

Measures of population differentiation (including clone-corrected values) for the Bowland and Worcestershire meadows are shown in Table 5.4. The F_{ST} value for the Worcestershire meadows was

significantly different from zero ($P = 0.01$ for both uncorrected and clone corrected data) as was the case for the Bowland meadows (see Chapter 4). Clone-corrected Pairwise F_{ST} values for the Worcestershire meadows are shown in Table 5.5. Values ranged from 0.022 to 0.298 whilst values for the uncorrected data (not shown) ranged from 0.033 to 0.441. A high proportion of clones was found in population BA and clone correction resulted in substantial reductions in pairwise F_{ST} values for this particular site and a reduction in the overall F_{ST} value. Overall F_{ST} and Hedrick's standardised G''_{ST} values were higher for the Worcestershire data than they were for the Bowland data indicating that there was greater population differentiation, and therefore less gene flow, between the study sites in Worcestershire than there was in Bowland. The overall values for the combined data were higher than either region but, after clone correction, the F_{ST} figure indicates that there is not a high level of population differentiation. Hedrick's G''_{ST} values were considerably higher than the F_{ST} estimates.

Table 5.4 Population differentiation estimates for the Bowland, Worcestershire and combined Bowland and Worcestershire data.

	F_{ST}	F_{ST} (cc)	G''_{ST}	G''_{ST} (cc)
Bowland	0.141	0.07	0.319	0.252
Worcestershire	0.219	0.153	0.357	0.294
Bowland and Worcs.	0.235	0.167	0.427	0.368

cc = clone corrected data

Table 5.5 Pairwise F_{ST} values for the Bowland meadows (clone corrected data)

	BA	BC	EM	HW	KP	LM	SA	SB
BC	0.126							
EM	0.022	0.133						
HW	0.169	0.298	0.125					
KP	0.111	0.102	0.086	0.168				
LM	0.132	0.170	0.101	0.162	0.067			
SA	0.087	0.127	0.043	0.140	0.088	0.143		
SB	0.033	0.122	0.033	0.122	0.061	0.058	0.095	
SW	0.058	0.112	0.052	0.184	0.095	0.071	0.114	0.052

Spatial analysis

The dbRDA revealed significant isolation by distance in the Worcestershire meadows (F = 1.202, df. = 2, P = 0.001). However, the relationship was a weak one with a low adjusted R^2 value of 0.001. The ordination of the Worcestershire sites (Fig. 5.3) does not show a clear pattern although site KP shows some separation from the other sites. This is the most geographically isolated site of the meadows included in the study. The Worcestershire sites form two groups, one located in the north east of the county and the other in the south west. When the sites are plotted according to these groups (Fig. 5.4) there is some separation along axis 2, although this axis is not significant in the permutation test (P = 0.323). The Y co-ordinates are significant which suggest there is a north-south pattern of isolation by distance.

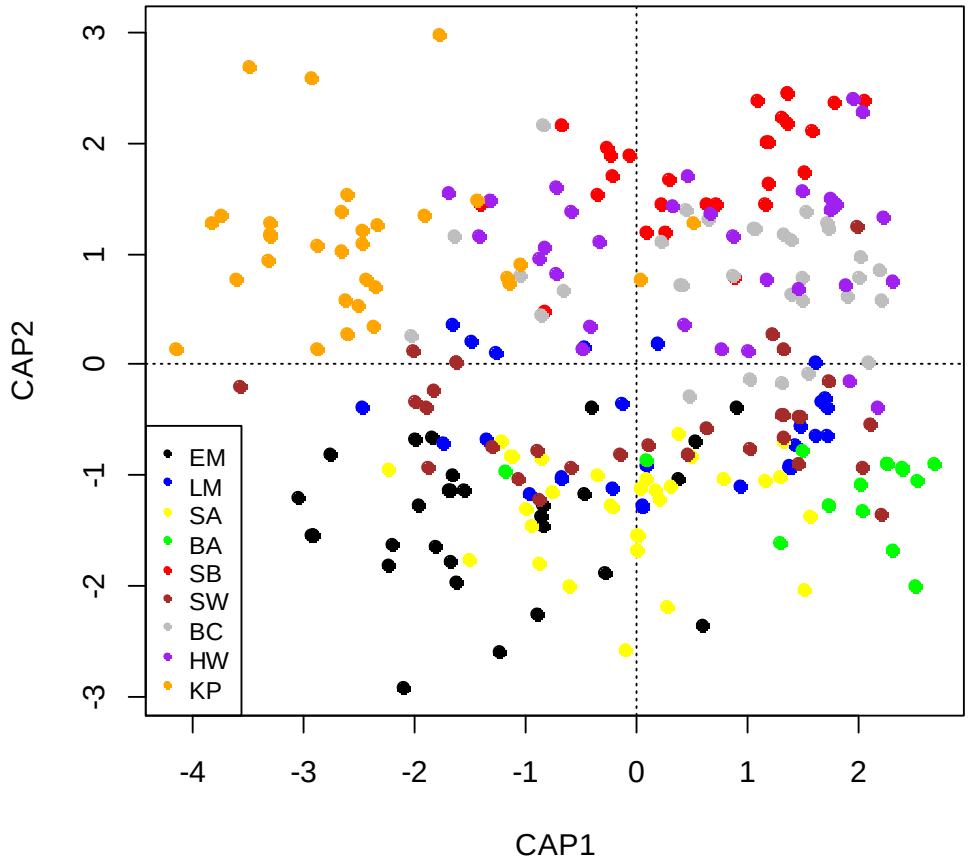


Fig. 5.3 Ordination of distance based redundancy analysis of Worcestershire data. Samples from meadow are represented by a different colour.

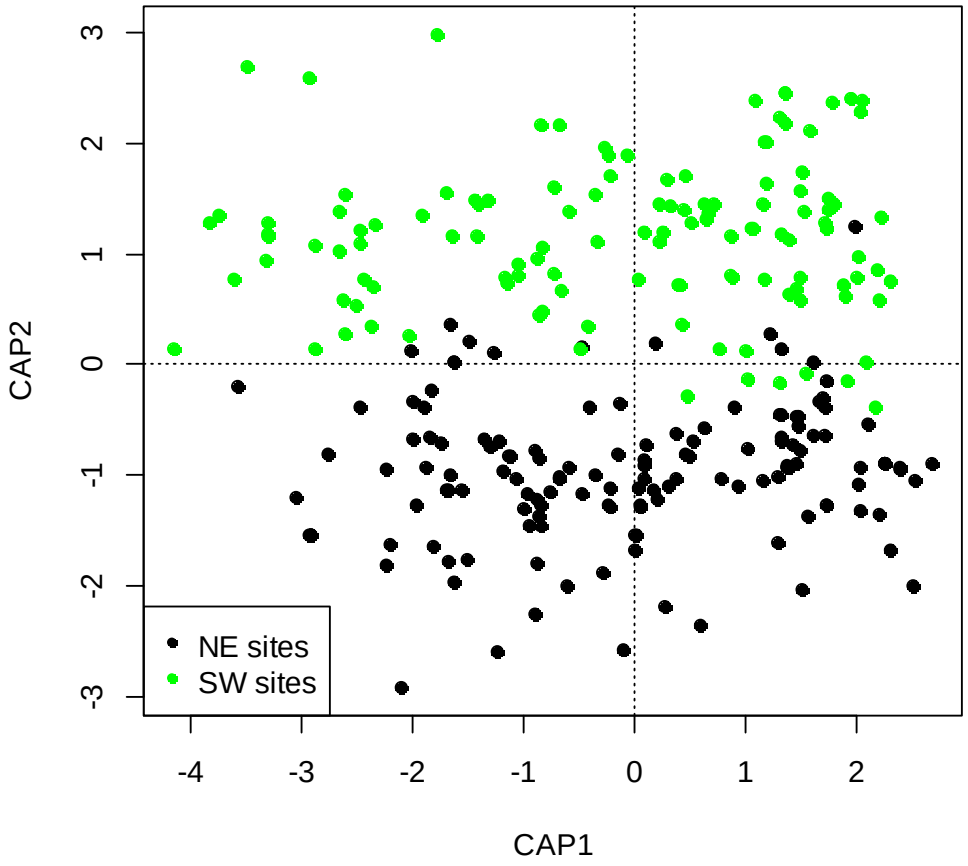


Fig. 5.4 Ordination of distance based redundancy analysis of Worcestershire data with plotting highlighting the two geographical groups of sites.

When the dbRDA was carried out on the combined Bowland and Worcestershire data there was also a significant pattern of isolation by distance ((F = 2.088, df. = 2, P = 0.001) although again with a low adjusted R^2 value of 0.004. The ordination plot (Fig. 5.5) shows separation between the two regions along axis 1 which was significant (P = 0.001) but there are also two clusters in the Worcestershire data which may reflect the fact that both the X and Y co-ordinates were found to be significant in the model (P = 0.001 in both cases). Identification of the Worcestershire sample points in the plot showed that the two clusters represent the north eastern and south western groups of meadows.

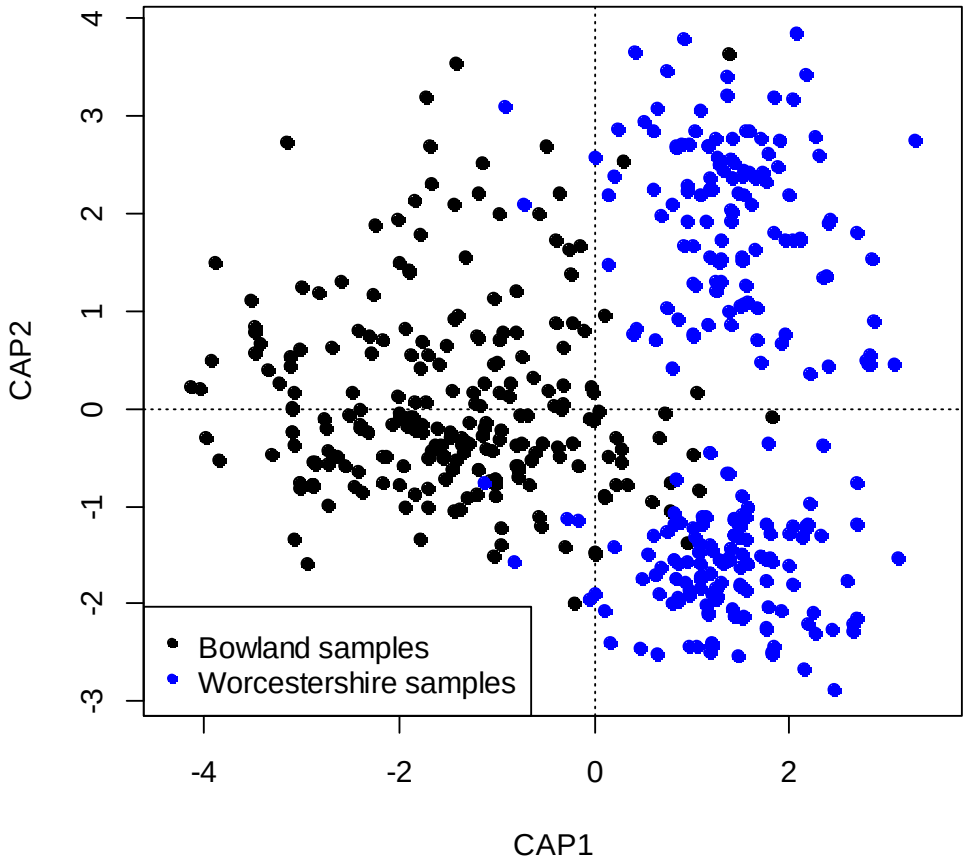


Fig. 5.5 Ordination of distance based redundancy analysis of Bowland and Worcestershire data. Showing separation between the two regions

The sPCA for the Worcestershire data revealed that there was significant global structure [max(t) = 0.040, P = 0.001] but did not show evidence of local structure [max(t) = 0.006, P = 1]. Some patterns of differentiation can be picked out in the colorplot (Fig. 5.6). Site BA and LM show some differentiation from all the other meadows and the three most south westerly and three most north easterly sites show some differentiation from each other. However site BC is similar to the north

easterly meadows. The contrast between the colours is not so marked as to suggest high levels of differentiation across the region but confirms that there is some evidence of isolation by distance.

The sPCA for the combined Bowland and Worcestershire sites indicated that there was both global structure [max(t) = 0.030, P = 0.001] and local structure in the data [max(t) = 0.006, P = 0.022]. The colorplot (Fig. 5.7) confirms the isolation by distance pattern identified by the dbRDA with the Bowland sites displaying red and green, and the Worcestershire sites displaying blue and green colours from the red, green and blue colour system. This suggests differentiation between the two regions although there are shared allelic patterns present in the data.

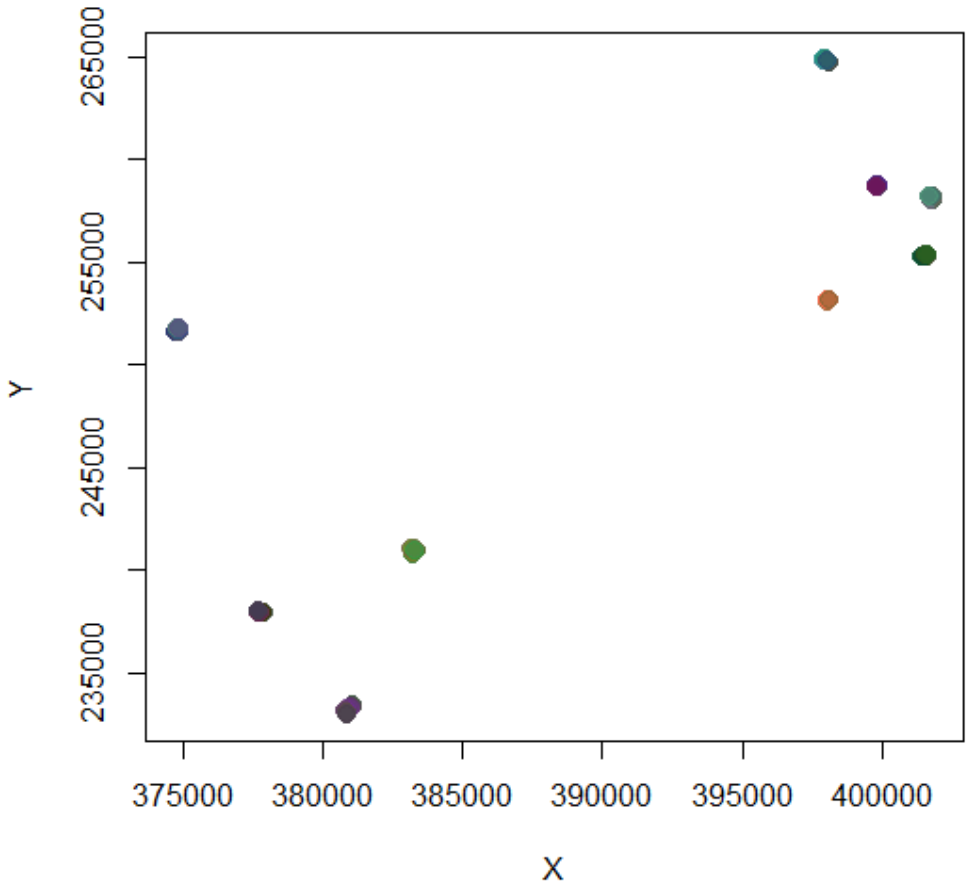


Fig. 5.6 Colorplot of sPCA of the Worcestershire sites. Colours are assigned using the red, green and blue colour system with greater contrast between colours representing greater genetic difference.

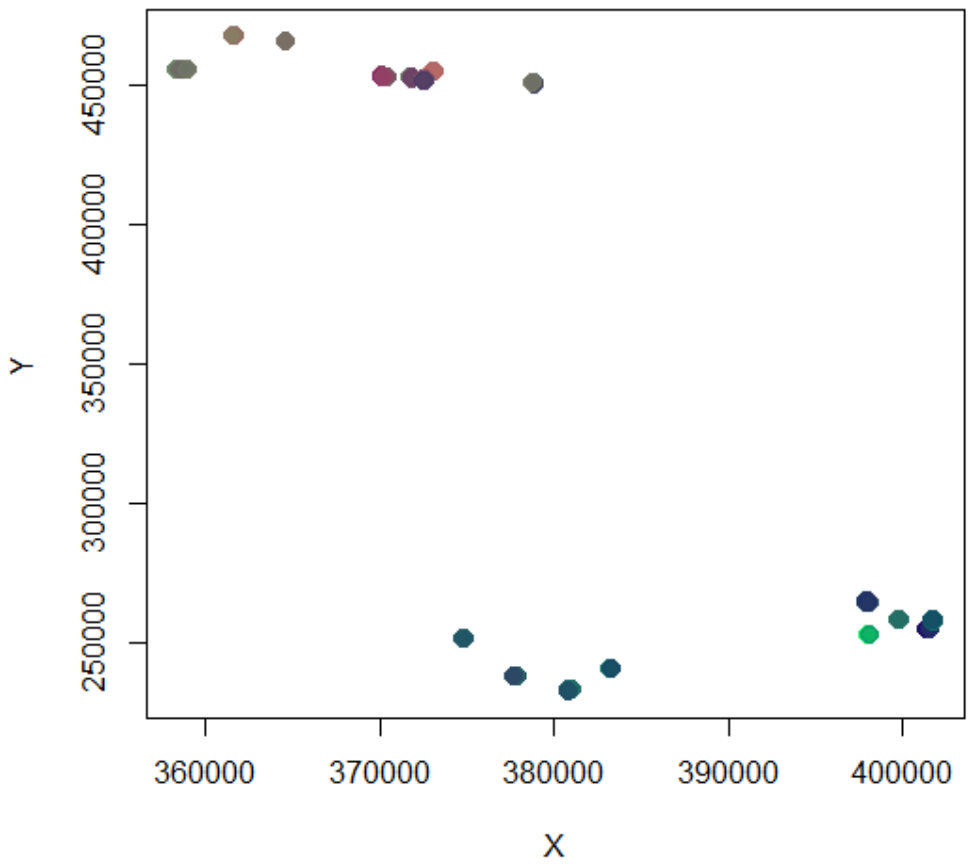


Fig. 5.7 Colorplot of the combined Bowland and Worcestershire data with Bowland sites in the top (northern) area of the plot and Worcestershire sites in the bottom (southern) area.

Discussion

This study has shown that the levels of genetic diversity in *R. minor* populations in the two regions were very similar despite contrasting topography and land use. The extent of gene flow between the study sites was lower in Worcestershire than it was in Bowland but in both regions populations were not highly differentiated and, although geographical distance between sites plays a part there may be other factors affecting differentiation. There is evidence to suggest that the Bowland meadows are differentiated from the Worcestershire sites.

Genetic diversity

There was very little difference between levels of heterozygosity and inbreeding in the two study regions. Estimates of effective population size were not calculated in this study but there was no significant relationship between site size in hectares and expected heterozygosity. There is a higher density of protected meadow sites in the Worcestershire sampling area than in the Bowland area but in both areas the meadows only make up a small fraction of the land area of the study regions. However, this fragmentation does not appear to have resulted in a particularly low level of genetic diversity. Inbreeding levels in both regions were equally high and this reflects the self-compatibility of *R. minor* which was discussed in Chapter 4.

Population differentiation and spatial analysis

The analysis of estimates of population differentiation in the two study areas indicates that there is less gene flow between the Worcestershire meadows than there is between the Bowland meadows. Samples were not collected from intermediate sites in Worcestershire so they cannot be included in the analysis as they were in Bowland. However, there are more protected meadow sites in the Worcestershire area which could be connected to the study sites, and most of the intermediate populations of *R. minor* were concentrated in one part of Bowland so their contribution to connectivity may be limited to that area.

All but one of the Worcestershire meadows are located at lower altitudes than the Bowland sites (see Table 5.1). Research into the effects of landscape features on gene flow has shown that altitudinal differences, even at substantially greater elevations than in Bowland, were not always a barrier to pollinators (Hargreaves *et al.*, 2015; Kamm *et al*., 2010). However, topography was found to be more influential, particularly in high mountain regions (Ægisdóttir *et al*., 2009; Thiel-Egenter *et al*., 2009).

In Bowland the meadows are mainly found in valleys separated by areas of higher ground but it may be that there were sufficient pollinator food sources across the whole area such that pollinator populations were not restricted by topography. The Worcestershire landscape has less varied topography although there are other potential physical barriers including major rivers such as the River Severn which divides the north eastern and south western sampling sites (see Fig. 5.1). Investigations into the barriers of landscape features to bumblebee movements have found that water bodies do limit movement to some extent (Jha, 2015; Lozier *et al*., 2013) as do artificial barriers such as roads and railways (Bhattacharya *et al*., 2003). In the area which was sampled in Worcestershire there are motorways, major roads and railways but there are only minor roads in the Bowland area.

It has been found that *Bombus* species (the principal pollinators of *R. minor*) were more abundant in landscapes with a diversity of habitats including semi-natural grasslands than they were in simple landscapes with large fields and no permanent pasture (Persson and Smith, 2013). These findings suggest that land use plays a more significant role in gene flow than topography or elevation in the two study regions but it is difficult to draw conclusions without more detailed investigations into all the possible landscape variables which could affect gene flow (Holderegger *et al*., 2010).

The pattern of population differentiation in the Worcestershire sites suggests that geographical distance is not the only influence on genetic distance. The sPCA plot (Fig. 5.6) shows that sites BA and SW are differentiated from the other sites. These are the smallest of the study sites and had the highest levels of inbreeding (BA: F_{IS} = 0.56 and SW: F_{IS} = 0.63) and lowest levels of observed heterozygosity (BA: H_o = 0.063 and SW: H_o = 0.062). However the correlation between site size and expected heterozygosity was not significant. It has been demonstrated that factors affecting population differentiation (including geographical distance, topography, abiotic and biotic environmental factors) can be effective at different scales (Mũnoz-Pajares *et al*., 2017). It is possible that there are local selection pressures which are affecting these sites differently from the others, a

possibility which may also apply to the Bowland sites in which a similar pattern of population differentiation was detected. Local adaptation has been detected in plant populations even where there is also evidence of gene flow (Gonzalo-Turpin and Hazard, 2009) but local adaptation varies among species and may be limited by high levels of selfing (Hamann *et al*., 2017; Linhart and Grant, 1996).

Population differentiation between the two regions was evident in the SPCA colorplot (Fig. 5.7) and there was a significant pattern of isolation by distance. This is not surprising given that there is a distance of approximately 200 km between the two study areas. However, this finding contrasts with results in other studies of other populations of *R. minor*. Houston and Wolff (2012) did not find isolation by distance in *R. minor* sampled from sites across the UK although there was isolation by distance when mainland European sites were included in the analysis. Talve *et al*. (2014) reported pairwise F_{ST} values higher than those in the Worcestershire or Bowland data when geographical distances between populations were 50 km or less. The isolation by distance and population differentiation shown in the combined Bowland and Worcestershire data suggests that there may be local adaptation among populations of *R. minor* in the two regions. The selection of seed sources for use in restoration projects from outside of the region could mean that the advantage of this local adaptation is lost. Restoration projects using *R. minor* seeds have been successful where local seed sources have been used but one of the reasons given for this was that the sites of the seed source and the restoration had a similar pH (Bullock and Pywell, 2005; Mudrák *et al*., 2014). These recommendations were based upon sowing experiments rather than analyses of genetic data but other studies have reported ecotypes of *Rhinanthus* species which appeared to be adapted to different localities or management regimes (Zopfi, 1993a, 1993b).

The importance of local adaptation in the context of restoration has been highlighted in a study by Durka *et al*. (2017) who found that populations of grassland species were genetically differentiated

across the country, and suggested that the use of seed transfer zones was an appropriate approach for grassland restoration. Seed transfer zones were also proposed by Holten *et al*. (2016) for a number of Alpine species in Norway, but both of these studies found that patterns of genetic differentiation varied from species to species, so it was recommended that more research was needed on other species used in restoration projects.

Conclusions and implications for conservation from the Bowland and Worcestershire studies

Comparisons of genetic diversity, estimated by expected heterozygosity, found that there was little difference between the two study areas. Levels of genetic diversity and gene flow, particularly in the Bowland region, indicate that conservation is being effective in maintaining viable meadow populations of *R. minor* in the two regions. Consistently high levels of inbreeding were reported with a large proportion of sampled individuals presenting as the same multilocus genotype indicating high rates of selfing. It would seem to be appropriate to take a precautionary approach to management until the reasons for high rates of selfing are better understood. This would entail action to increase population sizes and migration (i.e. gene flow) between meadows which would help to increase genetic variation, reduce inbreeding and, therefore, reduce the likelihood of inbreeding depression.

The contribution of intermediate sites in Bowland and the lower rate of gene flow in the Worcestershire area suggest that the landscape between meadows plays an important role in the genetic diversity of the *R. minor* populations now and in the future. There are more protected meadows in Worcestershire but it may be the intensively farmed landscape provides a barrier to pollinators, and opportunities to create intermediate populations in areas characterised by more intensive agriculture should be identified. At the same time the management of small grassland fragments such as those found on roadside verges, in church yards or community spaces as well as grasslands which are not protected should be maintained and enhanced.

The use of local seed sources would seem to be the best choice for meadow restoration projects following the combined analysis of the Bowland and Worcestershire sites. Local patterns of genetic structure in both areas complicate these findings particularly when some sites have a high proportion of clones. Such sites would not be ideal as donor sites in meadow restoration, certainly in terms of *R. minor*.

More research into seed sources for restoration is needed as is research about other meadow species (including perennial species, species with wind dispersal mechanisms for pollen and seeds, species which are not self-compatible) which could augment the findings from this study and better inform conservation in fragmented habitats.

CHAPTER 6. Conclusions

The aims of the study were to investigate long-term change in the community composition of mesotrophic semi natural grasslands and to assess whether the fragmented distribution of the grasslands had any influence on that change. The key findings were that change in community composition was associated with some management types, including hay meadows and that there were losses and gains of some meadow indicator species. The analysis of gene flow in *Rhinanthus minor* between sites in Bowland suggested that fragmentation was not having a major impact on populations of that species, but levels of gene flow were lower in the lowland grassland sites in Worcestershire. In general, hay meadow conservation appears to be maintaining the hay meadow community in the study area but some of the detail of the analysis indicates that more could be done to ensure the long-term viability of the hay meadow habitat, and a number of recommendations are set out at the end of this chapter.

Long-term change in mesotrophic semi-natural grasslands

It was expected that there would be change in grasslands which had been managed as hay meadows during the first survey but which had seen a change to more intensive management because the impact of agricultural intensification on plant diversity is well documented (Hodgson *et* al., 1999; Stoate *et al*., 2009). However, change in community composition was also recorded in sites which had been consistently managed as meadows; and grazed mesotrophic sites, most of which were not protected or in a higher tier AES, had not shown substantial change. The impacts of the management of grazed, mesotrophic lowland grasslands sites have not been given as much attention as those managed as meadows (Stewart and Pullin, 2008). Botanical interest was maintained on some sites but this was partly because the more species rich areas were in places which were more inaccessible to livestock. It may also be the case that change is slower in sites with a long history of extensive management (Cousins and Eriksson, 2002; Purschke *et al*., 2014).

The discussion about the use of quadrat and species list surveys in Chapter 2 illustrated the value of a systematic, quantitative approach to measuring change in species richness and diversity, along with the advantages of a whole site survey which records change in atypical parts of the site, and which detected the presence of rare species. In the UK protected site condition assessments use estimates of abundance of indicator plants which are the principal representatives of the target community with some flexibility to take account of species which may be of local importance (JNCC, 2004). However, it is unlikely that the resources allocated for this rapid assessment approach allow for a thorough, quantitative sampling of species abundance which could be subject to statistical analysis, or for an analysis of areas of the site which may support species of botanical interest not found in the main target communities. This level of monitoring would identify gradual changes in plant populations, picking up on losses before they became critical, and would provide a more complete record of the diversity of protected grassland sites. Current monitoring information could be compared with detailed baseline data, where it is available, such as survey data from the Lowland Grassland Inventory which was used in this study.

Species-rich hay meadows

Data from the two surveys indicated that conservation management of the meadows has maintained the meadow plant community and led to a decrease in negative meadow indicator species, but it has not prevented species turnover of positive indicator species, increased homogeneity of meadow vegetation and a net loss in the number of species recorded. Populations of plants can fluctuate from year to year due to environmental stochasticity which may be unrelated to longer-term trends (Shriver, 2016). However, some of the species which showed increases, eg, *Ranunculus repens*, and decreases, eg, *Alchemilla* spp., showed similar patterns of change over the same time period in another region of the UK (Starr-Keddle, 2014). It is possible that the standardised approach to meadow management, which is applied through AES prescriptions, may be maintaining a diverse

meadow community at the regional level, but could also be suppressing local site distinctiveness to some extent, and this could explain the change towards increased grassland generalists and greater homogeneity identified in Chapter 3. The management approach may need to be more flexible to take account of local differences in altitude, aspect, soil differences or other conditions which could affect phenology.

Impact of fragmentation and genetic analysis of *Rhinanthus minor* in the Bowland meadows

The moderate levels of genetic diversity and evidence of gene flow in the Bowland meadows indicated that conservation of hay meadows was maintaining populations of *R. minor*, and suggested that fragmentation did not appear to be having a significant effect. However, this analysis only considered one species. It may be that there is less gene flow among populations of other meadow indicator species, and this is an important area for further research which could explain some of the losses of these species. Plants respond to fragmentation differently and genetic diversity varies according to different traits such as dispersal mechanisms and breeding systems even in plants within the same habitat (Thiel-Egenter *et al*., 2009).

The high levels of inbreeding detected in both the Bowland and the Worcestershire populations of *R. minor* can be explained by the tendency towards selfing and by poor seed dispersal in this species. The reasons for high levels of selfing and its consequences for the long term viability of populations are less easily explained. There is evidence for negative impacts of inbreeding on fitness but the extent of inbreeding depression and the varying effects on different species are still being debated (Angeloni *et* al., 2011; Angeloni *et al.*, 2014; Pico and van Groenendael, 2007). Whilst this uncertainty exists it would seem appropriate to take a precautionary approach to the conservation of species which exhibit high levels of inbreeding by ensuring that population sizes are as large as possible, and taking action to maximise pollinator abundance.

Findings from the analysis of *R. minor* populations in sites other than the protected meadows indicated that these intermediate sites play an important role in gene flow. The pattern of isolation by distance in these sites suggested that they may be more vulnerable to isolation than the larger sites although the small sample sizes in some of the grassland fragments should be considered. Even so, the genetic similarity of the intermediate sites to their neighbouring protected sites provides some justification for the continued management and enhancement of additional populations of *R. minor* in order to maintain levels of gene flow.

The pattern of local population differentiation in Bowland was interesting, and further investigations into the historical management of some of the sites and possible environmental reasons for local adaptation would be needed to identify why these local differences have occurred. Such differences suggest that the choice of seed sources for restoration may require not only a local provenance but one in which donor and recipient site have similar physical characteristics.

Genetic diversity and population differentiation in the Worcestershire meadows

The comparison of *R. minor* populations in the Bowland and Worcestershire meadows revealed similarities in terms of levels of genetic variation and inbreeding but showed that there was less gene flow between the Worcestershire sites than there was in Bowland. This difference could be explained by the landscape matrix between the sites with features such as major roads, rivers and areas of more intensively farmed land presenting more of a barrier to pollinators in Worcestershire. An analysis of intermediate sites was not carried out in Worcestershire so it was not possible to compare this aspect of the research although there is a greater density of protected meadows in Worcestershire so it is likely that other populations of *R. minor* are present. It seems that there is a greater need for the restoration and enhancement of habitats which could function as part of the ecological network for meadow populations in an area like this where fragmentation appears to be having more of an impact.

The comparison of the two UK regions also provided evidence of isolation by distance and population differentiation between the Bowland and Worcestershire populations of *R. minor*. This has implications for the restoration of meadows using seed and indicates that seed sources from within the same region will be more genetically similar, and that the identification of seed transfer zones could be of value in restoration projects. However, a similar pattern of local differentiation was found in the Worcestershire meadows to that in the Bowland sites so, again the site conditions for both donor and recipient site should also be considered because local-scale adaptation may also be a factor.

Grassland restoration projects have been in place for over ten years in several parts of the UK, often utilising green hay strewing which requires that donor and recipient sites are in close proximity to minimise any seed shedding and wilting of the green hay during transport (Edwards *et al.*, 2007). Grassland restoration has become a key part of the conservation strategy for this habitat and is an option in the UK's Countryside Stewardship AES (Natural England, 2016). Assessment of the success of restoration has focused on species richness and diversity, and on functional traits in comparison with the donor site (Engst *et al*., 2016; Kirkham *et al*., 2012). Further research to investigate the potential for local adaptation by examining genetic similarity between donor and recipient site, and analyses of diversity and gene flow between donor and recipient sites over time would add a valuable measure of the long-term success of meadow restoration.

Recommendations for conservation and further research

- The conservation management of hay meadows should remain as a key element of grassland conservation both in the UK and in other locations which support this habitat. The current approach can be improved but it is being effective in maintaining a diverse grassland community.

- Monitoring of grassland sites should be adapted to involve the collection of quantitative data which can be analysed statistically, and to enable the recording of botanical interest in all areas of the site. Where it is available detailed baseline information such as that from the Lowland Grassland Inventory should be used to develop a source of long-term datasets which will become increasingly important in the analysis of large scale environmental impacts such as climate change. Data from similar monitoring activities would be of value across the network of Natura 2000 sites.
- The protection and conservation management of species rich, or moderately species rich grazed mesotrophic sites should be strengthened.
- Restoration and management of grassland sites including non-agricultural grasslands should be enhanced to build an ecological network and enhance gene flow. This landscape-scale approach is a particular priority in regions and in countries where grassland sites of high conservation importance are separated by areas of intensively farmed land and potential barriers to pollinators such as major roads.
- Local seed sources should be the first choice for grassland restoration projects and the donor sites should have similar site characteristics to the target recipient site.
- An investigation into the reasons for species change in grassland vegetation which is being managed for conservation is needed to help explain the widespread increases in *R. repens*, and *Juncus* spp. and declines in positive indicator species. Little attention has been given to the possibility that a standardised approach to grassland management is resulting in a loss of local distinctiveness and this should be examined in the UK and in other European countires.
- More research into the genetic variation and gene flow in meadow populations of species with contrasting longevity, breeding systems, pollen and seed dispersal mechanisms will extend our knowledge of the impacts of habitat fragmentation on different species and thus inform conservation priorities.

- More can be learned about the reasons for high levels of inbreeding in populations of *R. minor* including the possibility of a lack of pollinators, and this research could be extended to include other meadow species with similar breeding systems.
- Research into genetic variation and gene flow within and between donor and recipient sites in meadow restoration over time would provide another measure of the success of restoration. This could include sites which have been in restoration for 10+ years and more recently restored sites.

Concluding remarks

This study is the first to investigate long-term change in hay meadow vegetation in combination with an analysis of genetic diversity and gene flow. The long-term study revealed that the hay meadow community had been maintained at the regional level and the genetic analysis showed that the impact of fragmentation was limited in terms of gene flow, at least with respect to *R. minor*, which indicated that conservation is effective in the Bowland meadows. However the detail of the long-term analysis exposed the reductions in some meadow indicator species and a change towards greater homogeneity, and the possibility of local adaptation in some meadow populations suggests that management may need to take account of individual site conditions. At the same time the value of a network of grassland sites in addition to the key meadow sites was demonstrated. This requires a landscape-scale approach to conservation which is of particular importance in areas with fragmented habitats and a more intensively farmed or developed landscape.

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Appendix 1: Examples of positive indicator species for MG3, MG4 and MG5 grasslands (see Chapter 2)

Agrimonia eupatoria

Alchemilla spp.

Anemone nemorosa

Betonica officinalis

Centaurea nigra

Cirsium heterophyllum

Conopodium majus

Euphrasia spp.

Filipendula ulmaria

Galium verum,

Genista tinctoria

Geranium sylvaticum

Geum rivale

Lathyrus linifolius

Lathyrus pratensis,

Leontodon spp.

Lotus corniculatus

Oenanthe silaifolia,

Persicaria Bistorta

Pimpinella saxifrage

Polygala spp.

Potentilla erecta

Poterium sanguisorba

Primula veris,

Rhinanthus minor

Sanguisorba officinalis

Serratula tinctoria,

Silaum silaus

Succisa pratensis

Thalictrum Flavum

Trollius europaeus.

Source: JNCC 2004. *Common Standards Monitoring Guidance for Lowland Grassland Version February 2004*. Peterborough: Joint Nature Conservation Committee ISSN 1743-81

Appendix 2 Full species list for 35 mesotrophic grasslands

Acer pseudoplatanus L.
Achillea millefolium L.
Achillea ptarmica L.
Agrimonia eupatoria L.
Agrostis canina L.
Agrostis capillaris L.
Agrostis stolonifera L.
Ajuga reptans L.
Alchemilla filicaulis Buser
Alchemilla glabra Nygenf.
Alchemilla mollis (Buser) Rothm.
Alchemilla xanthochlora Rothm.
Allium ursinum L.
Alnus glutinosa (L.) Moench
Alopecurus geniculatus L.
Alopecurus pratensis L.
Anagallis tenella (L.) L.
Chamerion angustifolium (L.) Holub.
Chryosplenium oppositifolium L.
Circaea lutetiana L.
Cirsium arvense (L.) Scop.
Cirsium heterophyllum (L.) Hill
Cirsium palustre (L.) Scop.
Cirsium vulgare (Savi) Ten.
Comarum palustre L.
Conium maculatum L.
Conopodium majus (Gouan) Loret
Corylus avellana L.
Crataegus monogyna Jacq.
Crepis capillaris (L.) Wallr.
Crepis paludosa (L.) Moench
Cruciata laevipes Opiz
Cynosurus cristatus L.
Dactylis glomerata L.
Glechoma hederacea L.
Glyceria declinata Br.b.
Glyceria fluitans (L.) R. Br.
Heracleum sphondylium L.
Hieracum spp.
Holcus lanatus L.
Holcus mollis L.
Hyacynthoides non-scripta (L.) Chouard ex Rothm.
Hydrocotyle vulgaris L.
Hypericum pulchrum L.
Hypericum tetrapterum Fr.
Hypochaeris radicata L.
Ilex aquifolium L.
Impatiens glandulifera Royle
Juncus acutiflorus Ehrh. Ex Hoffm.
Juncus articulates L.
Juncus bufonius L.
Myrrhis odorata (L.) Scop.
Nardus stricta (L.)
Nasturtium officinale W.T. Aiton
Neottia ovata (L.) Bluff & Fingerh.
Odontites vernus (Bellardi) Dumort.
Ophioglossum vulgatum L.
Orchis mascula (L.) L.
Oreopteris limbosperma (All.) Holub
Oxalis acetosella L.
Parnassia palustris L.
Pedicularis palustris L.
Pedicularis sylvatica L.
Persicaria bistorta (L.) Samp.
Persicaria maculosa Gray
Petasites hybridus (L.) P. Gaertn., B. Mey & Scherb.
Phalaris arundinacea L.
Phleum pratense L.
Sagina spp.
Salix cinerea L.
Salix spp.
Salvia verbenaca L.
Sanguisorba officinalis L.
Schedonorus arundinaceus (Schreb.) Dumort.
Schedonorus giganteus (L.) Holub
Schedonorus pratensis (Huds.) P. Beauv.
*Scorzoneroides autumnalis (*L.) Moench
Scrophularia nodosa L.
Senecio aquaticus Hill
Serratula tinctoria L.
Silene dioica (L.) Clairv.
Silene flos-cuculi (L.) Clairv.
Sorbus aucuparia L.
Sorbus spp.
Stachys palustris L.

Anemone nemorosa L.

Angelica sylvestris L.

Anthoxanthum odoratum L.

Anthriscus sylvestris (L.) Hoffm.

Arctium minus (Hill) Berhn.

Arrhenatherum elatius (L.) P. Beauv. ex J. Presl & C. Presl

Athyrium filix-femina (L.) Roth

Avenula pubescens (Huds.) Dumort

Bellis perennis L.

Betonica officinalis L.

Betula pendula Roth.

Betula pubescens Ehrh.

Blechnum spicant (L.) Roth

Briza media L.

Bromus hordeaceus L.

Caltha palustris L.

Campanula rotundifolia L.

Capsella bursa-pastoris (L.) Medik.

Cardamine amara L.

Dactylorhiza fuchsia (Druce) Soó

*Dactylorhiza maculata (*L.) Soó

Dactylorhiza purpurella (T. & T.A. Stephenson) Soó

Danthonia decumbens (L.) DC.

Deschampsia cespitosa (L.) P. Beauv

Deschampsia flexuosa (L.) Trin.

Digitalis purpurea L.

Drosera rotundifolia L.

Dryopteris affinis agg.

Dryopteris carthusiana (Vill.) H.P. Fuchs

Dryopteris dilatata (Hoffm.) A. Gray

Dryopteris filix-mas (L.) Schott

Eleocharis palustris (L.) Roem. & Schult.

Elymus caninus (L.) L

Elytrigia repens (L.) Desv. ex Nevski

Epilobium hirsutum L.

Epilobium montanum L.

Epilobium palustre L.

Juncus bulbosus L.

Juncus compressus Jacq.

Juncus conglomeratus L.

Juncus effuses L.

Juncus inflexus L.

Juncus squarrosus L.

Koeleria macrantha (Ledeb.) Schult.

Lapsana communis L.

Larix spp.

Lathyrus linifolius (Reichard) Bässler

Lathyrus pratensis L.

Leontodon hispidus L.

Leucanthemum vulgare Lam.

Linum catharticum L.

Lolium perenne L.

Lonicera periclymenum L.

Lotus corniculatus L.

Lotus pedunculatus Cav.

Luzula campestris (L.) DC.

Pilosella officinarum F. W. Schultz & Sch. Bip.

Pimpinella saxifraga L.

Pinguicula vulgaris L.

Plantago lanceolata L.

Plantago major L.

Plantago media L.

Platanthera chlorantha (Custer) Rchb.

Poa annua L.

Poa pratensis L.

Poa trivialis L.

Polygala serpyllifolia Host

Polygonum aviculare L.

Polystichum spp.

Populus tremula L.

Potamogeton polygonifolius Pourr.

Potentilla anserina L.

Potentilla erecta (L.) Raeusch.

Potentilla reptans L.

Potentilla sterilis (L.) Garcke

Stachys sylvatica L.

Stellaria alsine Grimm

Stellaria graminea L.

Stellaria holostea L.

Stellaria media (L.) Vill.

Succisa pratensis Moench

Symphytum tuberosum L.

Taraxacum offincinale agg.

Taxus baccata L.

Teucrium scorodonia L.

Torilis japonica (Houtt.) DC.

Trifolium campestre Schreb.

Trifolium dubium Sibth

Trifolium medium L.

Trifolium pratense L.

Trifolium repens L.

Triglochin palustris L.

Trisetum flavescens (L.) P. Beauv.

Trollius europaeus L.

Cardamine flexuosa With.
Cardamine pratensis L.
Carex acutiformis Ehrh.
Carex binervis Sm.
Carex caryophyllea Latourr.
Carex demissa (Anderson) B.Schmid
Carex disticha Huds.
Carex echinata Murray
Carex flacca Schreb.
Carex hirta L.
Carex hostiana DC.
Carex lepidocarpa Tausch.
Carex leporina L.
Carex nigra (L.) Reichard
Carex pallescens L.
Carex panicea L.
Carex pilulifera L.
Carex pulicaris L.
Carex remota L.
Carex sylvatica Huds.
Centaurea nigra L.
Cerastium fontanum Baumg
Cerastium glomeratum Thuill.
Epilobium parviflorum Schreb.
Equisetum arvense L.
Equisetum palustre L.
Equisetum sylvaticum L.
Erica tetralix L.
Eriophorum angustifolium (Honck.)
Euphrasia spp.
Fagus sylvatica L.
Festuca ovina L.
Festuca rubra L.
X Festulolium loliaceum (Huds.) P. Fourn.
Ficaria verna Huds.
Filipendula ulmaria (L.) Maxim.
Fraxinus excelsior L.
Galium aparine L.
Galium palustre L.
Galium saxatile L.
Galium verum L.
Genista tinctoria L.
Geranium pratense L.
Geranium robertianum L.
Geranium sylvaticum L.
Geum rivale L.
Geum urbanum L.
Luzula multiflora (Ehrh.) Lej.
Lysimachia nemorum L.
Lysimachia nummularia L.
Malus sylvestris (L.) Mill.
Matricaria discoidea DC.
*Mentha aquatic*a L.
Mentha arvensis L.
Mercurialis perennis L.
Mimulus guttatus DC.
Molinea caerulea (L.) Moench
Montia fontana L.
Mycelis muralis (L.) Dumort.
Myosotis arvensis (L.) Hill
Myosotis discolor Pers.
Myosotis laxa Lehm.
Myosotis spp.
Myosotis scorpioides L.
Myosotis secunda Al. Murray
Poterium sanguisorba L.
Primula farinosa L.
Primula vulgaris Huds.
Prunella vulgaris L.
Prunus spinose L.
Pteridium aquilinum (L.) Kuhn
Pulicaria dysenterica (L.) Bernh.
Ranunculus acris L.
Ranunculus bulbosus L.
Ranunculus flammula L.
Ranunculus repens L.
Rhinanthus minor L.
Ribes spp.
Rosa arvensis Huds.
Rosa canina L.
Rosa spp.
Rubus fruticosus agg.
Rumex acetosa L.
Rumex acetosella L.
Rumex conglomeratus Murray
Rumex crispus L.
Rumex obtusifolius L.
Quercus spp.
Tussilago farfara L.
Ulex europaeus L.
Ulmus spp.
Urtica dioica L.
Vaccinium myrtillus L.
Vaccinium oxycoccos L.
Valeriana dioica L.
Valeriana officinalis L.
Veronica anagallis-aquatica L.
Veronica arvensis L.
Veronica beccabunga L.
Veronica chamaedrys L.
Veronica officinalis L.
Veronica scutellata L.
Veronica serpyllifolia L.
Vicia cracca L.
Vicia sativa L.
Vicia sepium L.
Viola palustris L.
Viola riviniana Rchb.

Appendix 3 Climate and nitrogen deposition data, Bowland.

Climate data for Stonyhurst weather station1985-2012 British National Grid Reference: SD684379	Nitrogen deposition data for neutral grassland habitat Slaidburn, Grid Reference: SD711530
Mean annual precipitation 1294mm Mean January temperature 4.0°C Mean July temperature 15.8°C	Deposition: 27.02 Kg N/ha/year for 2013-2015 Critical loads for low and medium altitude hay meadows: 20 - 30 Kg N/ha/year Critical loads for mountain hay meadows: 10 - 20 Kg N/ha/year

Climate data and nitrogen deposition data for the study area. Climate data source: Met Office, 2017; http://www.metoffice.gov.uk/public/weather/climate/gcw4rg5df. Nitrogen deposition data source: APIS2017 http://www.apis.ac.uk/queryLocationCheckbox-result?gridRef=SD711530&gridType=landranger&dropDownHabitat=Select+a+Habitat&pollutants%5B%5D=Acid&pollutants%5B%5D=NDep&submit=See+the+results

Appendix 4 Positive and negative meadow indicator species (see Chapter 3)

Positive indicator species	Negative indicator species
Alchemilla glabra	*Bromus hordeaceus*
Alchemilla xanthochlora	*Cirsium arvense*
Caltha palustris	*Dactylis glomerata*
Centaurea nigra	*Heracleum sphondylium*
Conopodium majus	*Holcus lanatus*
Euphrasia spp	*Juncus spp*
Filipendula ulmaria	*Lolium perenne*
Geranium sylvaticum	*Phleum pratense*
Geum rivale	*Poa trivialis*
Lathyrus pratensis	*Ranunculus repens*
Leontodon hispidus	*Rumex acetosa*
Leucanthemum vulgare	*Rumex obtusifolius*
Lotus corniculatus	*Stellaria media*
Lotus pedunculatus	*Trifolium repens*
Persicaria bistorta	
Rhinanthus minor	
Sanguisorba officinalis	
Scorzoneroides autumnalis	
Stachys officinalis	
Succisa pratensis	

Sources:

1. JNCC 2004. *Common Standards Monitoring Guidance for Lowland Grassland Version February 2004*. Peterborough: Joint Nature Conservation Committee ISSN 1743-8160.
2. Kirkham, F. W., Tallowin, J. R. B., Dunn, R. M., Bhogal, A., Chambers, B. J., Bardgett, R.D. 2014. Ecologically sustainable fertility management for the maintenance of species-rich hay meadows: a 12 year fertilizer and lime experiment. *Journal of Applied Ecology*, 51: 152-161.

Appendix 5

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Regional stability versus fine scale changes in community composition of mesotrophic grasslands over 25 years

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Regional stability versus fine scale changes in community composition of mesotrophic grasslands over 25 years

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Long-term studies of vegetation change in grasslands are important to our understanding of the ecology and management of grassland systems, especially for grasslands of high conservation value which have seen a drastic decline due to agricultural intensification and abandonment. This study investigated change over 25 years in 35 mesotrophic grassland sites which were described as species-rich at the start of the study period. Some sites had been consistently managed by mowing or grazing, while others had seen a change to more intensive management or to little or no regular management. Baseline data were available for both quadrat and species list surveys and repeat surveys were undertaken using the same methods on all 35 sites. Multivariate analysis using Non-metric Multidimensional Scaling revealed that the overall community composition was similar in the original and repeat surveys, but some differences were revealed when the sites were categorised by management type. The two survey methods provided different information about both the principal vegetation communities and about other aspects of the site including the presence of rare species. There were losses and gains of species of importance to conservation, with more losses than gains overall, and there was some evidence for species losses at sites which had been managed consistently for conservation. These changes may be linked to aspects of the management regime, isolation of sites or changes in soil fertility levels, but a greater understanding of the local and regional processes affecting diversity in mesotrophic grasslands is required to inform conservation management.

Keywords: long-term change, semi-natural grasslands, conservation, species loss

Introduction

Species-rich grasslands support a rich diversity of vegetation, but they are particularly vulnerable to change (Habel et al., 2013). In Europe, the maintenance of diversity in such grasslands usually requires an extensive agricultural management regime (Wesche et al., 2012), so the widespread adoption of intensive agricultural practices and the abandonment of more inaccessible or unproductive grasslands have resulted in the loss of the majority of species-rich grasslands in most European countries (Stoate et al., 2009). Studies which record long-term change in the remaining species-rich grasslands are rare but can make a valuable contribution to our understanding of ecological processes and help to inform management approaches (Magurran et al., 2010; Silvertown et al., 2010).

Some of the most diverse vegetation communities are found in calcareous grasslands and these habitats have been the subject of studies concerning change resulting from various influences and at different scales (Bennie et al., 2006; Diekmann et al., 2014; Van den Berget al., 2011). Although some attention has focused on change in hay meadows (Critchley et al., 2007; Homburger & Hofer, 2012), mesotrophic grasslands are less well studied, particularly those managed as pasture (Stewart & Pullin, 2008). However, mesotrophic sites can be botanically rich and may be more vulnerable to agricultural intensification than calcareous grasslands because of their higher levels of soil fertility (Hodgson et al., 2005). The management of semi-natural mesotrophic grasslands is often dependent on topography, with grazing dominating sites with steeper slopes while mowing for field-dried hay will be carried out on flatter ground (Andrieu et al., 2007). Community composition of grassland vegetation varies according to the management type (Klimek et al., 2007), so studies which consider sites under different management regimes provide additional important information about long-term change.

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Long-term, experimental studies such as the Park Grass experiment and the Steinach Grassland expermanagement treatments in grassland systems (Hejcman et al., 2014; Silvertown et al., 2010). Other approaches to investigating long-term change include re-visitation studies which consider various types of sites, sometimes located over a large geographical area. Such studies deliver valuable complementary information to that generated by the monitoring of experimental plots and provide an indication of change over a wider spatial scale. Re-visitation studies have revealed widespread change such as the effects on species richness in coastal vegetation communities around Scotland (Pakeman et al., 2016) and a loss of distinctive species in calcareous grasslands in sites across the UK (Bennie et al., 2006; Van den Berg et al., 2011). There are fewer studies of mesotrophic grasslands but Critchley et al. (2007) found a reduction in herb cover in species-rich hay meadows at a regional scale.

The present study investigated change in 35 mesotrophic grassland sites first surveyed in the 1980s and 1990s by the UK Nature Conservancy Council. The grasslands included sites which had been consistently managed as either hay meadows or as pastures. It also included sites originally managed as meadows but which had seen a change to more intensive management, and sites where there was no management or only occasional management. It would be expected that a change to more intensive management or to a lack of regular management would be more likely to result in corresponding changes in community composition, a relationship which has been widely discussed (Hodgson et al., 2005; Krause & Culmsee, 2013; Peco et al., 2005; Poschlod et al., 2005).

Re-visitation studies often use quadrat surveys to repeat previous vegetation surveys (Critchley et al. 2007; Meyer et al., 2015; Ross et al., 2012). Quadrats enable a standard, repeatable survey method although there is debate about optimum quadrat size and about inconsistencies in the estimation of percentage cover (Archaux et al., 2007; Kent, 2012). In the present study, repeat quadrat surveys were carried out, but baseline data were also available for site species lists for all of the sites included in the study. While the quadrat data account for the principal vegetation communities, whole site species lists can reveal information about the vegetation in atypical parts of a grassland site such as ditches, wetter areas and sloping banks, which were often less accessible to livestockor machinery and which can enhance the diversity of the vegetation across the site.

The statistical analysis of data obtained from studies which use stratified random sampling or quadrats placed subjectively in representative stands of vegetation will be more limited than that of data obtained from using an entirely random sampling design (Lajer, 2007). However, it is recognised that there is considerable value in the data from the numerous relevés which have been recorded over many years as part of phytosociological and other vegetation studies, provided that it is analysed and interpreted appropriately (Diekmann et al., 2007; Hédl, 2007; Lepš & Šmilauer, 2007). A similar approach should be taken with data collected from site species lists which can also be affected by surveyor bias but which can provide important information particularly where resources for surveys are limited (Gordon & Newton, 2006).

This study explored long-term change in the vegetation of 35 mesotrophic grasslands located across an upland region of north-west England. Unlike other revisitation studies, it combined a comparison of long-term change in grasslands with different management regimes and used data from two different survey methods.

The study addressed the following questions: (1) Has the overall community composition of grassland vegetation changed? (2) Are there differences in the extent of change between grasslands with different management types? (3)

Do the two survey methods provide contrasting information about vegetation change? (4) Which species are the main 'winners' and 'losers'?

Methods

Study area

The study was carried out in the Pennine region of north-west England. The study sites were located within an area of approximately 450 km^2 in the valleys of the Forest of Bowland which is an upland area situated at 53°58'N, 2°26'W (Fig. 1). The mean annual precipitation for the region is 1294 mm, mean January

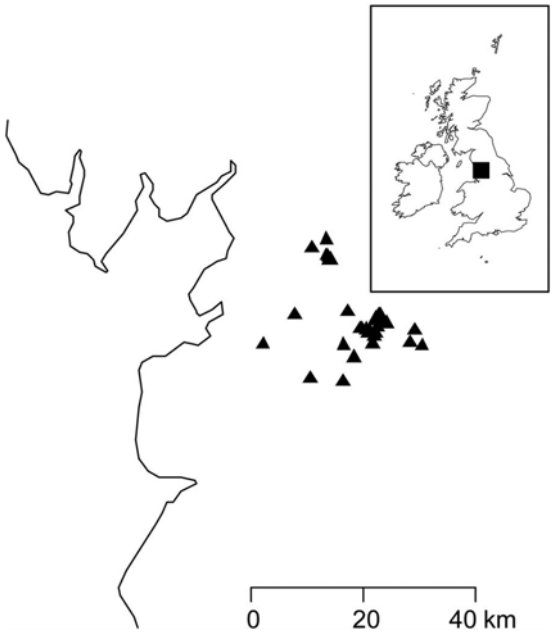

Figure 1 Location map.

temperature is 4.0°C and mean July temperature is 15.8°C (Met Office, 2016).

Site selection

Thirty-five grassland sites were identified where baseline survey data for both quadrat and species list surveys were available. The data had been collected in the 1980s and 1990s as part of a UK wide grassland survey (Blackstock et al., 1999). Part of this nationwide survey focused on mesotrophic lowland (i.e. below the moorland line or lower than approximately 300m above sea level) grasslands in Lancashire and it is this dataset that forms the baseline for the present study (Taylor, 1986). Grasslands in the original surveys were selected using existing Phase 1 habitat survey records and other local information and were chosen, because they were species-rich or moderately speciesrich. The surveys aimed to record sites which were important for conservation and to compare the botanical detail of sites with similar vegetation classifications. The study incorporated sites from the Forest of Bowland region with contrasting management regimes, including 14 sites which had been managed continuously as hay meadows since the original surveys were undertaken. Management details for these sites, such as earliest cutting dates, amounts of farmyard manure and dates of removal of grazing stock in the spring, can be linked to their inclusion in agri-environment schemes (AES) or designation as protected sites. Ten sites had been managed by grazing (cattle, sheep or a mixture of both). There were also six sites which were hay meadows at the time of the first survey but which had seen a change in management since the first survey was undertaken. The timing of the change is not known for all of the sites, but the current management is more intensive and involves either permanent grazing or cutting for silage rather than hay. The remaining five sites are no longer regularly cut or grazed but, again, details of the timings of the change are not known for all of the grasslands. The sites were located at altitudes varying from 60m to 280m above sea level. Sites varied in size from 0.2 ha to 11.59 ha (Table 1).

In the original surveys, the grasslands were classified under the UK National Vegetation Classification (NVC) as upland hay meadows MG3 Anthoxanthum odoratum-Geranium sylvaticum, floodplain meadows MG4 Alopecurus pratensis-Sanguisorba officinalis and lowland hay meadows or pastures MG5 Cynosurus cristatus-Centaurea nigra communities (Rodwell, 1992), although the majority of the surveys took place before the NVC was published and none of them was part of the NVC survey itself. These are the main communities, but some grasslands would also have supported, or still support, small areas of other mesotrophic examples. Most of the grasslands belong to the Triseto-Polygonion alliance or are

Table 1 Site details.

Site ID	Management type	Size (ha)	Altitude	Number of quadrats
AM	Former meadow	1.8	185	3
BG	Meadow	5.47	180	1
BG2	Meadow	2.2	180	1
BG3	Meadow	3.1	180	2
BS (3 fields)	Meadow	7.65	150	12
BS1	Meadow	2.3	180	2
BS3	Grazed	1.2	170	3
CB	Meadow	0.54	60	4
DH	Meadow	0.4	190	2
FH	Meadow	1.63	105	2
FHM	Meadow	3.33	201	4
HHL	Unmanaged	10.3	195	6
HHM	Unmanaged	0.3	105	2
LBL	Former meadow	1.7	140	2
LCB	Grazed	6.0	180	3
LCM	Meadow	5.26	190	2
LHBS	Grazed	0.76	130	2
LHG	Grazed	2.2	100	3
LRS	Unmanaged	0.2	120	2
LSM	Former meadow	1.1	230	1
LWM	Unmanaged	3.6	105	2
MM (2 fields)	Meadow	9.09	155	9
MM2	Grazed	0.7	160	3
NI	Meadow	2.09	125	6
NKM	Grazed	3.9	180	6
OWP	Grazed	0.3	160	6
PHB	Unmanaged	0.5	135	2
PP	Grazed	1.8	150	10
RH	Former meadow	1.8	80	2
SFP	Grazed	4.5	230	11
SM	Meadow	3.63	200	2
SPM	Grazed	1.4	280	6
TB (5 fields)	Meadow	11.87	155–180	7
TL	Former meadow	0.4	220	2
TSM	Former meadow	6.4	185	3

associated with alliances within the Molinio-Arrhenatheretea order (Rodwell et al., 2007).

Data collection

Repeat surveys (hereafter the second survey) were carried out using the original methods in the summers of 2012–2014. The original surveys (hereafter the first survey) followed Nature Conservancy Council guidance and involved the placing of 1×1m quadrats in areas deemed to be representative of the main vegetation communities (Smith et al., 1985). The guidance stated that the quadrats should be placed randomly within each vegetation community, although it was acknowledged that this would not always be possible, particularly in smaller stands of vegetation. In the meadow communities, a random sampling approach would be straightforward, but this might not have been achievable in some of the grazed sites where species-rich flushes and other smaller vegetation stands were surveyed.

Sketch maps of the locations of the first survey quadrats (see Smith et al., 1985 for an example map) were used to locate the quadrats in the second survey. The placing of the second survey quadrats followed the original approach by selecting areas representative of the main communities using the sketch maps and detailed descriptions of the vegetation to ensure that they were in the correct area of the site. The number of quadrats varied depending on the size and complexityof the sites (Table 1). In the meadows, quadratswere estimated to be within approximately 25m of the original location, although this would vary according to the size of site and number of quadrats. The grazed siteswere often more variable, with a mosaic of various vegetation communities which accounted for the higher number of quadrats in the original surveys, but in these sites the re-location of the quadrat was aided by descriptions of particular vegetation stands such as a species-rich flush or by proximity to a feature such as a stream. Presence and

abundance, using the Domin scale, of all vascular plants were recorded.

In addition to the quadrat surveys, the first survey involved the compilation of a species list covering all areas of the site on and within the site boundary (so vegetation in boundary hedges was included). Site boundaries included hedges, dry stone walls, post and wire fences and watercourses or ditches. None of these boundaries had been removed or re-positioned since the first surveys were undertaken. The NCC guidelines did not require surveyors to time the species list survey, but there was a requirement to include all of the vegetation communities on the site.

Data analysis

An exploratory approach to data analysis was taken, because it could not be assumed that random sampling methods had beenused for all of the quadrats or for the collection of species list data. To analyse differences in community composition, Non-metric Multidimensional Scaling (NMDS) ordinations were undertaken on the first and second surveys quadrat and species list data. The Domin scores recorded in the quadrat surveys were converted to percentage values using midpoint of each Domin category. The Bray Curtis dissimilarity matrix was used and the NMDS ordinations were carried out using the metaMDS function in the Vegan package in R (Oksanen et al., 2013). The NMDS ordinations examined community composition by year and then separate ordinations were carried out on the quadrat data to investigate the four management types, i.e. meadows, grazed sites, former meadows with more intensive current management and sites which had little or no management.

To investigate patterns in community composition revealed by the two survey methods (quadrat surveys and species list surveys), the quadrat data were first converted from abundance data to presence/absence data so that they were analysed in the same format as the species list data. NMDS ordinations for the two survey types were then compared using Procrustes analysis in the Vegan package (Oksanen et al., 2013). Procrustes analysis is used to investigate the extent to which there is a fit between one ordination or dataset and another and produces a correlation score, indicating the extent of the fit based on the distances between the sampling points or sites. A low scorewould indicate that there was little similarity between the two ordinations and vice versa. Protest does return P values, but large datasets can affect the validity of P values and it is recommended that the r value is more useful in interpreting the outcome of the test (Oksanen et al., 2013).

To analyse species losses and gains, species were ranked according to the frequency at which they had been recorded by site in the first and second surveys in both quadrats and species lists.

In the UK, guidance is issued for the monitoring of protected mesotrophic grassland sites (JNCC, 2004). The guidance lists species for each grassland community which are considered as positive indicators whose presence is indicative of favourable conservation status. These indicator species are used to evaluate the conservation value of particular grassland communities and to address whether the target vegetation community is being maintained or not. The frequency of positive and negative indicator species by site was compared for the first and second surveys. Indicator species are listed in Appendix 1.

To assess whether there was any indication of change in functional type in the increased and decreased species, mean values for Ellenberg Indicator Values (EIVs) for the British plants for light (L), moisture (F), reaction (R) and fertility (N) were calculated for the most increased and decreased species (Hill et al., 1999). Weightings were not used for the EIVs, because there were no abundance data for the species lists. Ellenberg values can give an indication of changes in environmental conditions and are useful as a proxy measure when no soil data are available, as was the case for these surveys. Calculations of Grime's C-S-R plant strategy scores, using the tool developed by Hunt et al. (2004), were also undertaken and assigned to the most increased and decreased species. The modal C-S-R type was calculated.

All analysis was carried out in R version 3.1.2 (R Development Core Team, 2014).

Results

In the quadrat survey, the total number of species recorded across all 35 sites was 152 from the first survey and 144 from the second survey (a decrease of

5.26%). In the species list survey, the totals were 268

pattern in the sites with little or no management (Fig. 4c and d).

The two quadrat and species list NMDS ordinations were not found to have a similar configuration. The Protest permutation test returned an r value of 0.27 which suggested that there was little correlation between the two

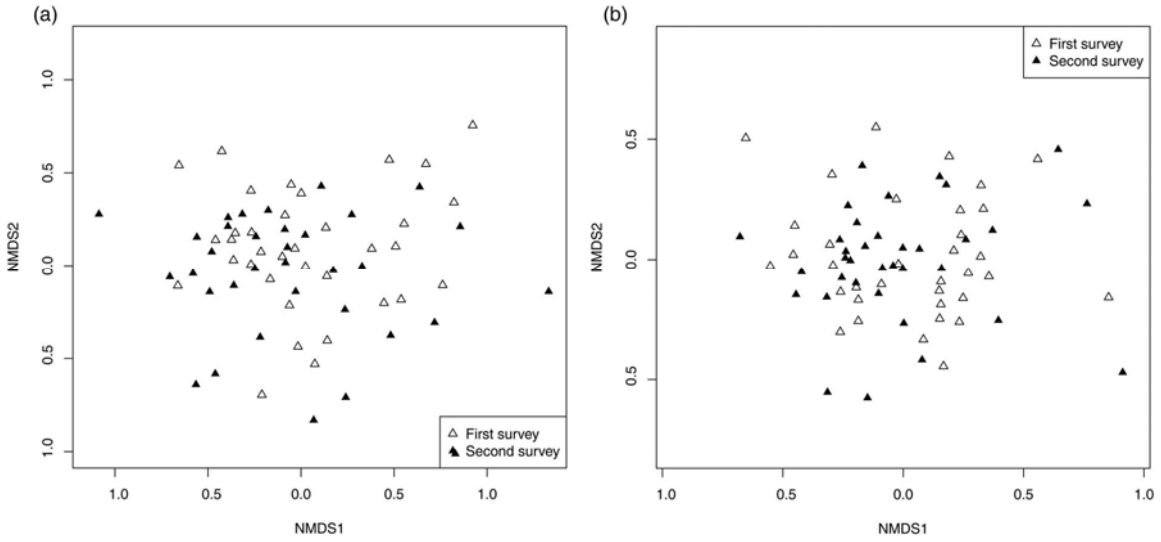

Figure 2 (a) NMDS ordination of quadrat data for the first and second surveys. Points represent grassland sites. Stress = 0.22. (b) NMDS ordination of species list data for the first and second surveys. Points represent grassland sites. Stress = 0.22.

from the first survey and 229 from the second survey (a decrease of 14.55%).

The NMDS ordination plots do not show a distinct separation of survey sites by year for either the quadrat data or the species list data (Fig. 2a and b), indicating that there is little difference in overall community composition between the two survey years.

The NMDS plots for management types show that there is some differentiation between the survey years (Figs. 3 and 4). In the meadow sites, there is some separation along both axes for the quadrat data (Fig. 3a), with less difference between the two years in the species list data (Fig. 3b). In the grazed sites, the differences between the two years are less distinct although two or three sites in each plot appear to have a different community composition than the majority of the grazed sites. Figure 4a and b show that change has taken place in sites which were managed as hay meadows at the time of the first survey and are now more intensively managed for silage or by permanent grazing. There is a less distinct

ordinations. This result indicates that the two survey methods revealed contrasting results in terms of community composition.

More species had shown a decrease than an increase in terms of the number of site records (Tables 2 and 3). Table 2 shows the 25 species which showed the greatest decrease for both the quadrat and species list data (see Appendix 2 for a full species list). Eleven of the 25 species appear in both the quadrat and species list data. Examples of species which are regarded as positive indicators for mesotrophic grassland were found in both sets of data (e.g. Anemone nemorosa and Leontodon hispidus). Some negative indicator species were also found to have decreased (e.g. Dactylis glomerata). Species showing the most increases in site records are shown in Table 3. Fewer species had shown a substantial increase in site records, particularly in the quadrat data, but there were some examples of positive (e.g. Euphrasia spp.) and negative indicator species (e.g. Juncus effusus).

The analysis of increased and decreased species showed that there were higher EIV scores for light

and moisture in the increased species for both quadrat and species list data (Table 4). For reaction (pH), there was a lower score in the increased quadrat species than the decreased but a higher score in the increased species list species. There was a similar pattern for fertility scores with a lower score for the increased quadrat species when compared with the most decreased species. The fertility score for the most increased species for the species list data appears to be substantially greater than that for the most decreased species, although this was not tested statistically. Modal types for C-S-R signatures were different in the most increased species than the decreased species for both the quadrat data and the species list data, with a shift away from the stress-tolerator type.

Discussion

Analysis of community composition

Taken as a whole, the community composition of the 35 grassland sites had remained similar between the two survey years based on both the quadrat surveys and the species list surveys. This finding does not reflect the accounts of significant change in other grassland re-visitation studies (Bennie et al., 2006; Bühler & Roth, 2011). The overall finding of limited change in the mesotrophic grasslands included in the present study may suggest that they are more resilient to change than other grassland habitats where the negative impacts of atmospheric nitrogen deposition and other sources of eutrophication on species richness or diversity have been greater (Stevens et al., 2010; Van den Berg et al., 2011). Differences in the responses of grassland habitats to nitrogen deposition have been identified, but the results are influenced by several factors including the baseline nutrient levels of the grasslands in the study (Maskell et al., 2010) and the varying effect of reduced or oxidised forms of nitrogen on the component species of acidic, calcareous or mesotrophic grassland communities (Van den Berg et al., 2016).

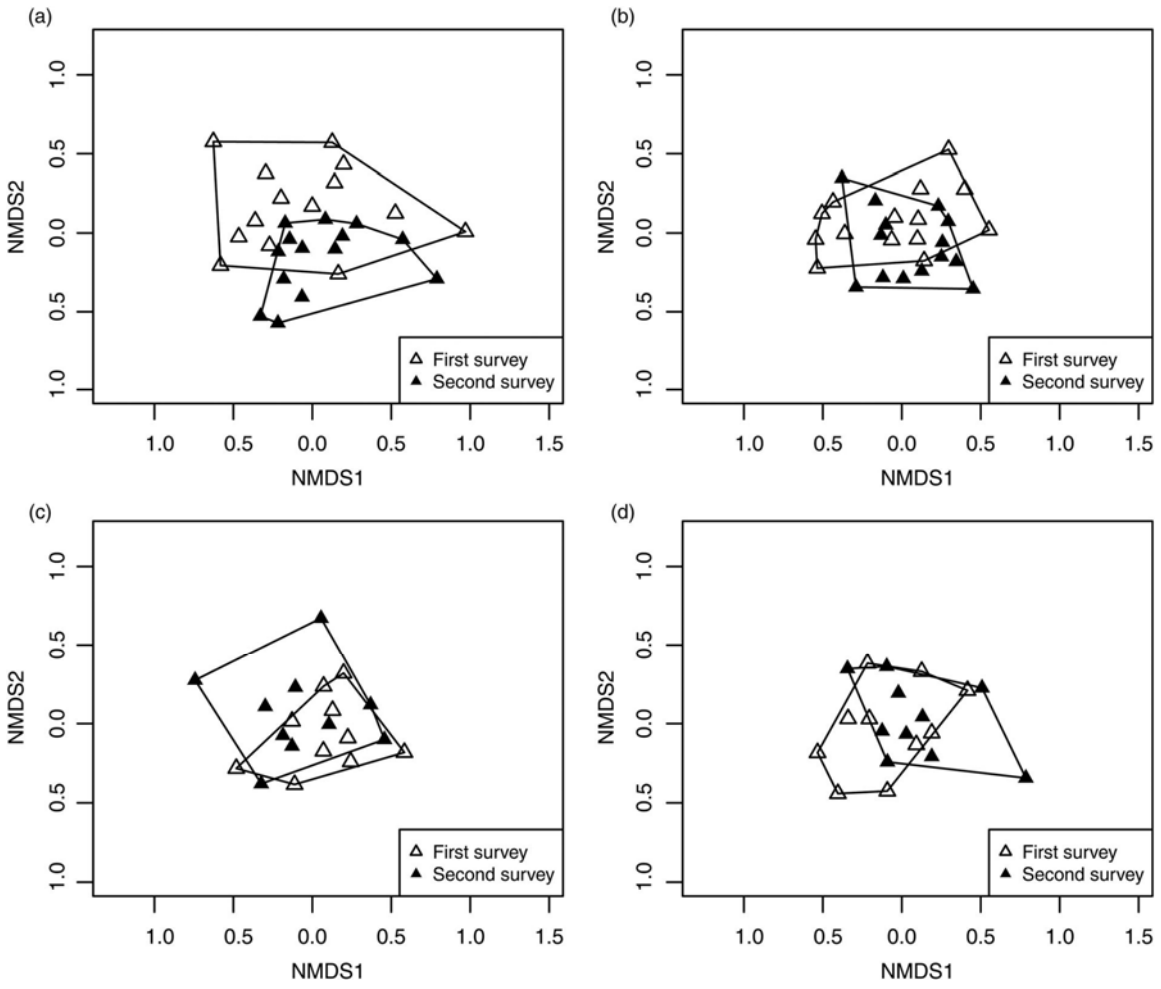

Figure 3 NMDS ordinations of quadrat and species list survey data for the two survey years by management type. Plot a shows quadrat data for meadow sites (stress = 0.18), plot b shows species list data for meadow sites (stress = 0.17), plot c shows quadrat data for grazed sites (stress = 0.19) and plot d shows species list data for grazed sites (stress = 0.19).

Analysis by management type

Analyses of the community composition of the meadow sites in the first and second surveys indicated that there had been more change identified through the quadrat surveys than by the species list surveys. All of the meadow sites are subject to statutory protection and/or higher tier AES and have similar management regimes. It is possible that a particular aspect of this management regime is the reason for this change to the meadow community rather than a more widespread environmental impact which may have been more likely to have an impact on vegetation across the site. Another factor could be the effect of the isolation of populations of plants within the main meadow community, since these sites are few and have a fragmented distribution. Detailed investigations of drivers of change are outside the scope of this study, but more research into the significance of potential influences such as management, the fragmented distribution of sites, site location factors (e.g. altitude, aspect) as well as wider environmental factor such as nitrogen deposition would be valuable.

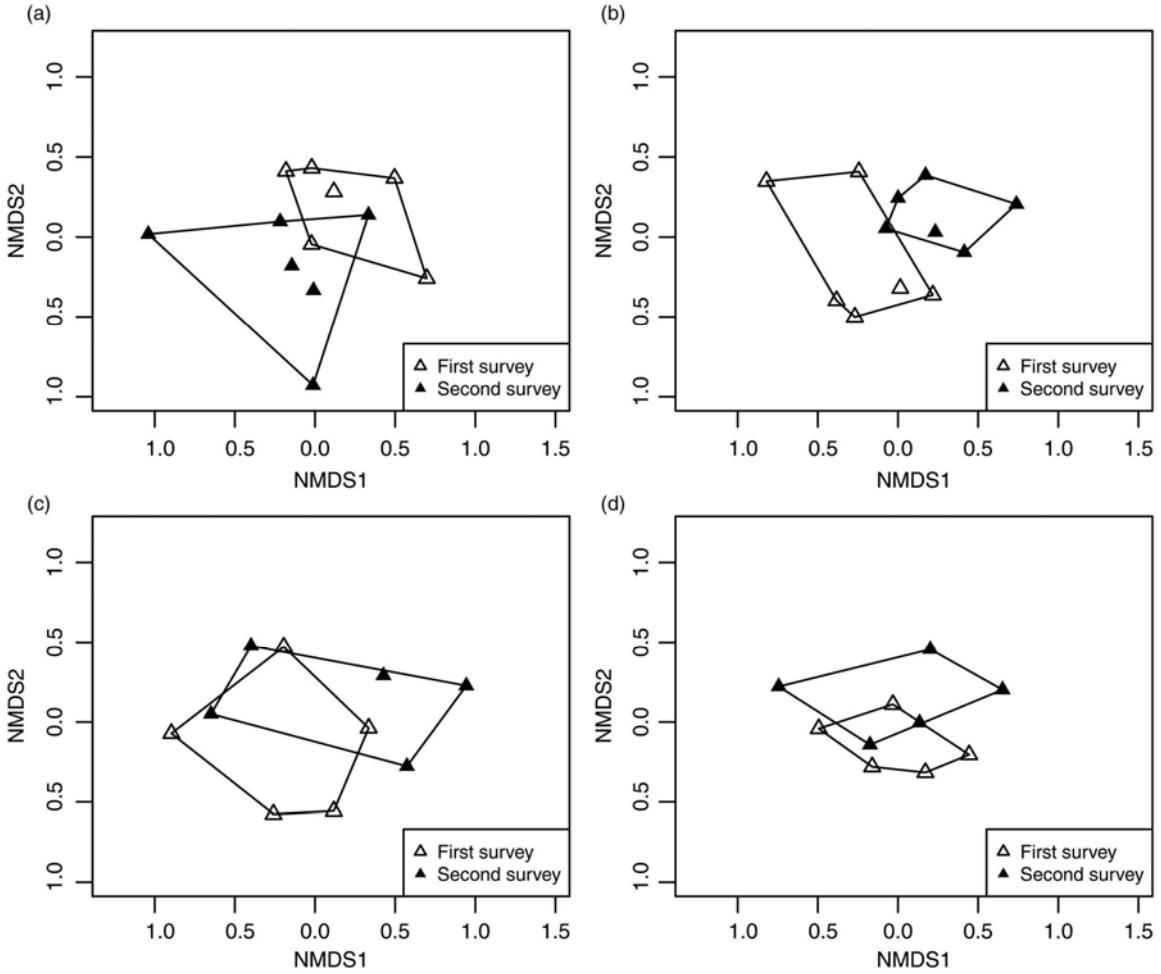

Figure 4 NMDS ordinations of quadrat and species list survey data for the two survey years by management type. Plot a shows quadrat data for former meadow sites which are now more intensively managed (stress = 0.13), plot b shows species list data for intensively managed former meadow sites (stress = 0.13), plot c shows quadrat data for sites with little or no management (stress = 0.10) and plot d shows species list data for sites with little or no management (stress = 0.10).

There is less clear evidence of change in the grazed sites, although two sites from the second survey in the quadrat plot (Fig. 3c) show some separation from the others. One of these sites included plants associated with mire communities. The other site was being affected by encroachment of the woodland adjacent to it and supported woodland as well as grassland species when the second survey was undertaken. In the species list plot, (Fig. 3d) some of the first survey sites show a degree of separation. Losses of species richness were recorded on these sites during the second survey which would account for differences in community composition. It was

expected that overall there would be less similarity in the grazed sites due to the greater variation in terms of topography, hydrology and soil conditions, and in their management where livestock type, stocking density and timing of grazing could all influence the vegetation from site to site. However, this does not appear to be the case according to the data collected for this study. Unlike the meadows, only three of the pasture sites are protected, with the others either in lower tier AES schemes, which are less demanding in the management of grasslands for conservation (Natural England, 2013a, 2013b), or not part of any AES agreement, but this lack of a conservation framework for management does not appear to have led to significant change. The NMDS ordinations are valuable for comparing community composition across several sites, but they are less useful in detecting fine scale changes which could be occurring within these sites. Hutson (1999) stressed the importance of scale in patterns of vegetation diversity and demonstrated that local conditions can influence regional diversity, but such influences can be complex and are dependent on the scale of the study and the type of community.

It was expected that there would be significant change in the vegetation of the grasslands which had seen a change to a more intensive management regime, since it is well documented that grasslands require regular low intensity management to maintain botanical diversity (Cuelmans et al., 2013; Klimek et al., 2007; Snoo et al., 2012). These sites do appear to have experienced the most change, although there is not a complete separation of the two survey years. However, the small sample size of the changed sites

Table 2 Decreases in number of records of species at grassland sites for quadrat survey data and species list data.

Quadrat survey data				Species list survey data			
Species	Number of site records (1st survey)	Number of site records (2nd survey)	Decrease in site frequency	Species	Number of site records (1st survey)	Number of site records (2nd survey)	Decrease in site frequency
Luzula campestris	20	6	−14	Achillea ptarmica	22	5	−17
Poa pratensis	14	2	−12	Poa pratensis	18	1	−17
Alchemilla glabra	15	4	−11	Ficaria verna	17	1	−16
Centaurea nigra	27	16	−11	Luzula campestris	25	12	−13
Achillea ptarmica	12	2	−10	Achillea millefolium	25	13	−12
Dactylis glomerata*	20	10	−10	Cardamine pratensis	29	18	−11
Phleum pratense*	17	7	−10	Angelica sylvestris	16	6	−10
Bellis perennis	18	9	−9	Ajuga reptans	13	4	−9
Bromus hordeaceus	13	4	−9	Anemone nemorosa	9	0	−9
Ficaria verna	12	3	−9	Avenula pubescens	14	5	−9
Conopodium majus	19	11	−8	Alchemilla xanthochlora	8	0	−8
Hypochaeris radicata	14	6	−8	Cirsium vulgare*	8	0	−8
Leontodon hispidus	13	7	−6	Festuca ovina	16	8	−8
Plantago lanceolata	33	27	−6	Leontodon hispidus	19	11	−8
Prunella vulgaris	19	13	−6	Ranunculus bulbosus	9	1	−8
Trifolium repens*	31	25	−6	Bromus hordeaceus	14	7	−7
Ajuga reptans	6	1	−5	Centaurea nigra	30	23	−7
Alchemilla xanthochlora	5	0	−5	Conopodium majus	24	17	−7

Anemone nemorosa	5	0	−5	Phleum pratense*	21	14	−7
Juncus inflexus*	6	1	−5	Plantago major	12	5	−7
Lathyrus pratensis	22	17	−5	Vicia cracca	21	14	−7
Ranunculus bulbosus	5	0	−5	Agrostis capillaris	34	28	−6
Sanguisorba officinalis	21	16	−5	Cerastium glomeratum	7	1	−6
Achillea millefolium	13	9	−4	Heracleum sphondylium	18	12	−6
Agrostis canina	4	0	−4	Tussilago farfara	8	2	−6

The 25 most decreased species are shown. Species in bold are examples of positive indicators for UK mesotrophic grasslands and species with an asterisk* are examples of negative indicators (JNCC, 2004).

means that the results have to be treatedwith some caution. Reference has already been made to the variations in site characteristics in pastures and the distinctiveness of individual sites was also a feature of the changed and unmanaged sites. For example, one heavily grazed former meadow site had retained manyof the indicator species in the short sward, while another with similar management had new records of some meadow indicators. Information on the dates for the changes in management was not available, but research has

Table 3 Increases in number of records of species at grassland sites for quadrat survey data and species list data.

Quadrat survey data				Species list survey data			
Species	Number of sites recorded (1st survey)	Number of sites recorded (2nd survey)	Gain in site frequency	Species	Number of sites recorded (1st survey)	Number of sites recorded (2nd survey)	Gain in site frequency
Ranunculus repens	20	31	11	Alopecurus geniculatus	8	19	11
Euphrasia spp.	8	13	5	Galium palustre	6	17	11
Galium palustre	3	7	4	Juncus effusus*	15	25	10
Glyceria declinata	0	3	3	Urtica dioica*	14	24	10
Lotus corniculatus	13	16	3	Alopecurus pratensis	14	23	9
Luzula multiflora	2	5	3	Dactylorhiza fuchsii	10	18	8
Myosotis discolor	5	8	3	Juncus articulatus*	12	20	8
Alopecurus geniculatus	1	3	2	Ranunculus repens	28	35	7
Juncus effusus*	5	7	2	Myosotis discolor	12	18	6
Trifolium dubium	1	3	2	Poa trivialis	26	32	6
Vicia cracca	8	10	2	Euphrasia spp.	13	17	4
Trifolium medium	0	1	1	Galium aparine	2	6	4
Triglochin palustre	0	1	1	Glyceria declinata	1	5	4
Urtica dioica*	1	2	1	Poa annua	4	7	3
Vaccinium oxycoccos	0	1	1	Rumex obtusifolius*	15	18	3

The 15 most increased species are shown. Species in bold are examples of positive indicators for UK mesotrophic grasslands and species with an asterisk* are examples of negative indicators (JNCC, 2004).

Table 4 Mean Ellenberg Indicator Values (EIV) and C-S-R types for most increased and decreased species.

	Mean EIV				
	Eb L	Eb F	Eb R	Eb N	Modal C-S-R type
Most decreased species (quadrat data)	6.92	5.36	5.88	4.36	CSR
Most increased species (quadrat data)	7.07	6.50	5.36	4.29	CR
Most decreased species (species list data)	6.80	5.36	5.88	4.56	CSR
Most increased species (species list data)	6.93	6.43	6.29	6.21	CR

Eb L = light; Eb F = moisture; Eb R = reaction; Eb N = fertility. C = competitor; S = stress-tolerator; R = ruderal.

shown that site management history and other small scale factors, such as the current and past land use history of neighbouring sites as well as hydrological and soil conditions, can all have a significant effect on current species diversity and composition (Gustavsson et al., 2007; Kalusová et al., 2009; Reitalu et al., 2009). There is some evidence of change in the unmanaged sites although again the small sample size must be taken into account. The lack of regular management appeared to have had an impact on species with a lower growth habit such as Trifolium repens and Luzula campestris. This is consistent with a study by Pavlu˚ et al. (2011) which compared mown and unmanaged grasslands and reported similar results where graminoids and forbs with a short growth habit occurred less frequently in unmanaged plots.

Findings from quadrat and species list surveys

In total, more species were recorded in the species list surveys, which was expected because the quadrat survey data are a sub-sample of the whole site. In the changed sites, for example, some species not found in the main sward had been retained on steeper banks at the edges of the sites. Some rare and uncommon species were picked up in the species list survey including Primula farinosa, Platanthera chlorantha, Cirsium heterophyllum and Genista tinctoria, which have very few local records and are declining at the national level (Greenwood, 2012; Preston et al., 2002). The comparison of the data resulting from the two survey methods showed that they had identified differences in terms of community composition. These differences can be explained by the fact that the species list survey required that all vegetation communities on the site were included. Features, such as streams, ditches, areas close to a woodland boundary, gateways where there was evidence of eutrophication or more heavily trampled areas or small areas of acid or calcareous grassland which were not part of the quadrat survey, were present on some sites. It is acknowledged that the effect of the sampling methods used should also be considered here. Surveyor bias and subjectivity will have some influence, particularly in the compilation of the species lists, so care is needed in the interpretation of the results. Ideally, monitoring of long-term change should minimise sampling bias and error, and the approach taken by Critchley & Poulton (1998) illustrates the value of precision and accounting for the optimum monitoring scale for different species. However, most revisitation studies aim to replicate the methods of the original survey, so there is a trade-off between the value of the long-term data and the limitations imposed by the original survey design.

Species losses and gains

The changes in species records suggest a mixed picture in terms of the maintenance of the target plant communities of species-rich mesotrophic sites. There were losses of some grassland species of conservation interest such as Alchemilla glabra which was found only in quadrats on 4 sites in the second survey (compared to 15 in the first), although losses recorded in the species list survey were less widespread (a decrease of 21 to 17 sites). Gains in positive grassland indicator species were also recorded (e.g. for Euphrasia spp.), but there were fewer gains than losses. There were losses and gains in site records for negative indicator species such as Dactylis glomerata which saw a substantial reduction in the quadrat survey and Urtica dioicawhich increased from 14 to 24 sites in the species list survey.

Some losses of positive indicators would be expected, given the change to more intensive management in the former meadow sites, but they may also reflect the impact of particular management prescriptions in sites which are being managed for conservation. For example,

Ranunculus repens was recorded on all 35 sites and in most of the quadrats in the second survey. A study which investigated the control of R. repens and Juncus spp. (which also showed a large increase) found that early summer mowing dates were effective in reducing the abundance of R. repens, while an autumn cut reduced Juncus spp. (Marriott et al., 2003). These cutting dates would not be permitted under AES management prescriptions for meadow sites.

The higher mean Ellenberg N score for the increased species in the species list data is mainly a result of increases in species such as Urtica dioica, Rumex obtusifolius and Galium aparine which have Ellenberg N scores of 8 or 9. The Ellenberg N values in the increased species in the quadrat data were lower, which could suggest that the species list scores were a result of localised increases of particular species. These species are also competitor species, so their increases also influence the C-S-R scores. Ellenberg values and C-S-R strategies are useful, but they may not take into account some of the more subtle changes in the dynamics of these grassland communities, changes which may also be too fine-scale for a regional analysis of community composition in all of the 35 sites in this study. Suding et al. (2005) found that while species richness always declined when soil nitrogen increased, there were varying responses among different plant traits and habitat types. Rare species and nitrogen-fixing forbs were vulnerable to increases in fertility but so too were some perennials, because of their conservative growth strategies in comparison to other more rapidly growing species which used the increased nitrogen more effectively. Conservation approaches which enhance rare species, but also take account of the dynamics of different functional groups, will require a greater understanding of these fine-scale processes and how they relate to regional patterns of diversity, along with further long-term study to monitor their effectiveness.

Conclusion

The community composition of the 35 grassland sites had not seen a marked change at the regional level over the period of study. This is in contrast to the substantial changes noted in other re-visitation grassland studies. However, important finer-scale change was identified and grassland management had an influence on plant communities. Different survey methods provided contrasting information about the grassland sites, and the combination of quadrat surveys and species lists can provide valuable information about key vegetation communities and other aspects of the site such as the presence of rare species. There were losses and gains of positive indicator species and changes in negative species, but overall there were more losses than gains. This is a concern and more research is needed to understand why such losses are occurring, particularly in sites which are protected and managed for conservation

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